CHAPTER ONE

FUNCTIONAL NUCLEAR ARCHITECTURE
STUDIED BY MICROSCOPY:
PRESENT AND FUTURE

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Abstract

In this review we describe major contributions of light and electron microscopic approaches to the present understanding of functional nuclear architecture. The large gap of knowledge, which must still be bridged from the molecular level to the level of higher order structure, is emphasized by differences of currently discussed models of nuclear architecture. Molecular biological tools represent new means for the multicolor visualization of various nuclear components in living cells. New achievements offer the possibility to surpass the resolution limit of conventional light microscopy down to the nanometer scale and require improved bioinformatics tools able to handle the analysis of large amounts of data. In combination with the much higher resolution of electron microscopic methods, including ultrastructural cytochemistry, correlative microscopy of the same cells in their living and fixed state is the approach of choice to combine the advantages of different techniques. This will make possible future analyses of cell type- and species-specific differences of nuclear architecture in more detail and to put different models to critical tests.

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1. Introduction

Impressive progress could be witnessed during the last 10 years in the field of epigenetics. Based on multifaceted studies of covalent histone modifications and their interplay with DNA methylation sites, as well as histone variants and chromatin remodeling events, molecular biologists have attempted to decipher what may be called the chromatin language (Guil and Esteller, 2009; Jiang and Pugh, 2009; Mercer et al., 2009; Munshi et al., 2009; Strahl and Allis, 2000; Varga-Weisz and Becker, 2006). Understanding this language, its species-specific modifications and the mechanisms involved in its cell type-specific expression, has become a central part of ongoing efforts to understand genome and nuclear functions. In comparison with the widely used term histone code (Jenuwein and Allis, 2001; Strahl and Allis, 2000), the term chromatin language emphasizes that histone modifications do not provide a code in the sense of DNA triplets coding for a certain amino acid, but must be read and understood in the context of other modifications of the chromatin environment. As the fertilized egg progresses through development and translates its information into a multitude of cell fates, one genome can generate many “epigenomes” (European Epigenome Network of Excellence: What is epigenetics? http://www.epigenome-noe.net/aboutus/epigenetics.php). Whereas researchers in
epigenetics attempt to understand the chromatin language, we argue that understanding the functional implications of a multitude of epigenomes will not be possible by epigenetic studies alone, but requires in-depth studies of nuclear architecture as well (Fig. 1.1).

Nuclear architecture represents the highest level of structural order and information content of the epigenome. Detailed knowledge of the dynamic nuclear architecture is indispensable for understanding gene regulation and other nuclear functions (Bartova et al., 2008; Chuang and Belmont, 2007; Fakan and van Driel, 2007; Fraser and Bickmore, 2007; Gasser, 2002; Kosak and Groudine, 2004; Lanctot et al., 2007a; Misteli, 2007; Pederson and Singer, 2006; Rippe, 2007; Spector, 2003; Takizawa et al., 2008; Trinkle-Mulcahy and Lamond, 2008). Notably, the possible extent of functionally important cell type- and species-specific differences is still unknown. Knowledge of evolutionarily conserved structural principles and species-specific peculiarities of the nuclear architecture is still in an unsatisfactory state. Although it is now well established that changes of nuclear phenotypes, including changes of higher order chromatin arrangements, take place during differentiation of somatic cell types within a developing organism, the functional implications of such changes are not known. Nor do we know which differences of the nuclear architecture may play an important role in the deregulation of genes in cancer cells (Ye et al., 2007). No consensus has been reached with regard to the common principles of nuclear architecture shared by all eukaryotes. Contradictory models of nuclear architecture (discussed below) are a reflection of this fact. Repositioning of genes, from repressive to transcriptionally favorable nuclear compartments and vice versa, proposed by some of these models apparently correlate with changes of transcriptional activities. In addition, we are confronted with the task to explore the whole range of cell type- and
species-specific differences and understand their functional implications. Attempts to analyze the extent of functionally important Brownian or directed chromatin movements are still in their very beginning, not to speak of the mechanisms which assure that any nucleus in an organism displays the correct higher order structure at the right place and the right time.

Within the size limits of this review a full coverage of the present state of research in nuclear architecture was not possible. We refer readers to an addendum, where we provide a few pertinent references to topics excluded from further consideration. Topics excluded from this review include the nucleosome (Olins and Olins, 2003; Woodcock, 2006) and its role in epigenetic gene regulation, insulators, and boundaries that delimit differentially regulated loci (Capelson and Corces, 2004; Ishii and Laemmli, 2003; Lunyak, 2008), the disputed concept of nuclear matrix and higher order chromatin organization (Berezney and Coffey, 1974; Bode et al., 2003; Elcock and Bridger, 2008; Hancock, 2000; Kaufmann et al., 1986; Malyavantham et al., 2008; Pederson, 2000; Razin et al., 2007; Zink et al., 2004), higher order chromatin structures achieved by chromatin self-assembly (Albiez et al., 2006; Misteli, 2001; Poirier and Marko, 2002), structure and function of the nucleolus (Boisvert et al., 2007; Hernandez-Verdun, 2006; McKeown and Shaw, 2009), the nuclear envelope, its pore complexes, and interaction with chromatin (Akhtar and Gasser, 2007; Kalverda et al., 2008; Maco et al., 2006; Prunuske and Ullman, 2006), composition and molecular biology of nuclear bodies and inclusions (Bernardi and Pandolfi, 2007; Gall, 2003; Morris, 2008; von Mikecz, 2009), interchromatin granules or splicing speckles (Fakan, 2004; Lamond and Spector, 2003; Thiry, 1995a), topography and functional roles of telomerases (Gasser et al., 2004; Hediger and Gasser, 2002). We also excluded nuclear architecture and chromosome dynamics in meiotic cells from this review (Kleckner, 2006; Scherthan et al., 2007; Vazquez-Nin et al., 2003), as well as topics with established or potential clinical applications, such as laminopathies (Dechat et al., 2008; Goldman et al., 2005), nuclear architecture in senescent cells (Funayama and Ishikawa, 2007; Malatesta et al., 2003; Narita, 2007; Oberdoerffer and Sinclair, 2007) and cancer cells (Londono-Vallejo, 2008; Zaidi et al., 2007; Zink et al., 2004).

We attempt to provide an overview of microscopic studies exploring the functional nuclear architecture. In the first part we summarize what is known, and more important, what is still not known about the in situ topography of chromatin and various other nucleoplasmic components. We point out the necessity to investigate nuclear architecture at the single cell level, including the various compactions and lengths of chromatin fibers or loops and the dynamics of higher order chromatin arrangements. In the second part it is our goal to provide molecular biologists with the background necessary to better understand the complex world of quantitative microscopy and image analysis. Molecular biologists may be at a loss to judge
the possibilities and limitations of microscopic approaches and consequently the reliability of quantitative microscopic data (Ronneberger et al., 2008). We also review recent breakthroughs in fluorescence microscopy (FM), which have opened access to the observation of structures at nanoscale dimensions (<100 nm), and discuss how these new approaches can be most fruitfully combined with the superior resolution of electron microscopy (EM). In this context we argue for the necessity of correlative microscopic studies of nuclear architecture following the same cell, first in the living and then in the fixed state, from the level of conventional and advanced 3D (space) and 4D (space–time) FM to the EM level.

Advanced microscopic studies of nuclear architecture would be impossible without uncounted probes for the visualization of specific chromatin and nonchromatin domains in a multicolor format. Examples include DNA probes of different complexity for the visualization of chromatin structure and order at all levels ranging from entire chromosome territories (CTs) to single genes by fluorescence in situ hybridization (FISH) under conditions which conserve the spatial nuclear arrangement as much as possible at this level of resolution (Cremer and Cremer, 2001, 2010; Solovei et al., 2002). Three-dimensional (3D) RNA FISH has allowed not only to identify patterns of the global nuclear topography of transcription but also to determine the nuclear positions of individual transcriptionally active genes (Capodieci et al., 2005).

The possibility to express specific nuclear proteins tagged with fluorescent proteins in combination with fluorescence recovery after photobleaching (FRAP) technology has revolutionized our insights into the dynamics of protein movements in the cell nucleus (Lippincott-Schwartz et al., 2003; Misteli, 2001). Expression of nuclear proteins tagged with fluorescent proteins and the construction of transgenes with DNA recognition sites that bind to specific fluorescently labeled proteins has provided a boost for studies of higher order chromatin arrangements and gene position in nuclei of live cells (Gerlich et al., 2003; Janicki et al., 2004; Kanda et al., 1998; Robinett et al., 1996; Walter et al., 2003b).

Recently, Rothbauer et al. (2006) generated fluorescent, antigen-binding nanobodies (chromobodies) that can be expressed in living cells and represent a new targeting tool (Rothbauer et al., 2006). The generation of many more in vivo probes for multicolor live-cell imaging of various nuclear structures will be one of the most important needs of microscopists from molecular biologists in the near future. Considering the necessity that such probes should not interfere with nuclear functions in unexpected ways, this task is a very demanding one.

Novel molecular methods have made possible the genome-wide mapping of protein–DNA and DNA–DNA interactions. Although a detailed discussion of these techniques is beyond the scope of this review, we wish to emphasize their unique possibilities for studies of the nuclear
architecture and complementarity to microscopic approaches. An approach, which allows the construction of genome-wide maps of DNA sequences interacting in vivo with DNA binding proteins, was introduced by van Steensel and Henikoff (2000). This method is based on the expression of a fusion protein, which consists of the protein of interest and the DNA adenine methyltransferase (Dam). DNA sequences carrying methylated adenines in the vicinity of the protein of interest are amplified by a methylation-specific PCR protocol and identified by hybridization to microarrays (Vogel et al., 2007). A recent breakthrough, achieved with this elegant method, was the genome-wide characterization of DNA sequences interacting with the lamina underpinning the nuclear envelope in Drosophila melanogaster and human cells (Guelen et al., 2008; Pickersgill et al., 2006).

Another approach with a great potential for nuclear architecture studies, called “chromosome conformation capture” (3C), was introduced by Dekker et al. (2002). The method is based on in situ formaldehyde cross-linking of DNA–DNA interactions. The original 3C approach has prompted numerous improvements to determine nonrandom spatial interactions between CTs at the level of specific DNA segments in cis (sequences within the same CT) and trans (sequences from different CTs) (Babu et al., 2008; Dekker, 2008; Dostie et al., 2007; Hu et al., 2008; Lin et al., 2009; Ling et al., 2006; Lomvardas et al., 2006; Noordermeer et al., 2008; Nunez et al., 2009; Osborne et al., 2007; Sexton et al., 2009; Simonis and de Laat, 2008; Tiwari et al., 2008; Williams and Flavell, 2008; Zhao et al., 2006). The combination of circular chromosome conformation capture (4C) with DNA microarrays (Göndör et al., 2008; Schoenfelder et al., 2009) or massively parallel sequencing (Lieberman-Aiden et al., 2009) has allowed for the first time mapping of DNA–DNA interactions in cis and trans at a genome-wide level. Although DNA interactions in cis were found to abound, significant interactions in trans were detected in agreement with other studies cited above. This wealth of data, often substantiated by 3D FISH assays, has provided strong support for long-range interactions in cis, that is, between genes located many megabases (Mbs) apart on the same chromosomes, or trans, that is, between genes located on different chromosomes. To date, numerous spatial interactions of CTs were reported in cis and trans (Babu et al., 2008; Ling et al., 2006; Lomvardas et al., 2006; Noordermeer et al., 2008).

Notably, 3C assays provide “high-throughput” possibilities to screen epigenomes globally for interactions in cis and trans. Their very principle, however, prevents their application at the single cell level. Present assays typically require some 10–20 million cells to screen for nonrandom interactions between DNA sequences. In cultured cell populations and in tissues consisting of a variety of different cell types, 3C assays cannot pinpoint statistically significant increases of specific DNA interactions to a specific cell type. Furthermore, these assays are complex and demand strict controls.
in order to escape the danger of false positive or negative findings (Dekker, 2006; Simonis et al., 2007). High-throughput assays and microscopic approaches are complementary (Simonis and de Laat, 2008). Quantitative microscopic approaches are needed to validate the findings of 3C assays and they are indispensable to determine the cell types in complex tissues, which show chromatin interactions in cis and trans. More importantly, microscopic approaches are the only way to reveal the entire structure of nuclear components and to determine their topography with respect to each other. Microscopic techniques have now been developed to the point, where they are capable to resolve interactions at the molecular level in the living cell.

2. Historical Background

Since the late 19th century (Boveri, 1888; Rabl, 1885), an uncounted number of microscopic studies have appeared on numerous aspects of nuclear structure, yet methodology to investigate nuclear architecture at high resolution and in three dimensions became only available during the second half of the 20th century. Starting in the 1960s, first insights into the functional compartmentalization of cell nuclei were achieved by transmission electron microscopy (TEM) of nuclei (Fakan and Bernhard, 1971; Monneron and Bernhard, 1969; Olins et al., 1983; Smetana and Hermansky, 1963), followed during the 1980s by advanced 3D light microscopy methods (Agard and Sedat, 1983; Belmont et al., 1987; Popp et al., 1990). The basic repeating unit of chromatin, known since 1975 as the nucleosome (Oudet et al., 1975), was described in the 1970s (Kornberg and Thomas, 1974; Olins and Olins, 1974; Woodcock et al., 1976a,b). Studies of chromosome arrangements at this time were restricted to mitotic cells except for a few special cases (Comings, 1980).

We focus this brief historical account on the discovery of CTs described in detail elsewhere (Cremer and Cremer, 2006a,b). To our knowledge, Carl Rabl (1885) and Eduard Strasburger (1905) were the first scientists who argued for a territorial organization of chromosomes in animal and plant nuclei, respectively. Theodor Boveri introduced this term in a seminal publication from 1909 arguing that each chromosome retains its individuality during interphase and occupies a distinct part of the nuclear space (Boveri, 1909). Notably, this hypothesis fell into oblivion during the 1950s–1980s, when only few researchers still adhered to this seemingly outdated concept, while most seemed to be content with the idea that chromatin fibers and loops intermingle in the nuclear space like a dish of spaghetti. Based on chromatin staining with a modified Giemsa banding technique, Stack et al. (1977) described CTs in nuclei from Allium cepa root tips and a Chinese hamster cell line, whereas the Cremer group obtained...
evidence for CTs in nuclei of diploid Chinese hamster cells with the help of laser–UV–microirradiation experiments ($\lambda = 257$ nm) (Cremer et al., 1982a,b, 1984; Zorn et al., 1976, 1979). These experiments were based on the following rationale (Cremer and Cremer, 2006b; Meaburn and Misteli, 2007): (1) The microbeam was used to produce UV–damaged DNA within a small part of the nucleus (about 5% of the total nuclear area). (2) The damaged DNA was traced from interphase to mitosis and identified on metaphase chromosomes. (3) Decisively different patterns of damage distribution on metaphase chromosomes were predicted for a territorial and a nonterritorial organization of interphase chromosomes (Cremer et al., 1982a). Two procedures were followed for the in situ detection of damaged DNA: Cells microirradiated in G1 were pulse-labeled with $^3$H-thymidine. Incorporation of the radioactive compound into microirradiated DNA was obtained during excision repair of DNA photolesions. Alternatively, microirradiated DNA was visualized by indirect immunofluorescent staining with antibodies raised against UV–damaged DNA. The latter approach made it possible to follow chromatin micro-irradiated at any time during interphase (including S-phase) from interphase to mitosis (Cremer et al., 1980, 1984; Hens et al., 1983). As predicted for Boveri’s early CT hypothesis, the results demonstrated that only a few mitotic chromosomes showed heavily labeled segments (Rabl, 1885; Strasburger, 1905). These segments constituted the parts of neighboring CTs hit by the microbeam.

In situ hybridization experiments made it possible to visualize CTs in mammalian interphase nuclei (Cremer et al., 1988, 1993; Lichter et al., 1988; Manuelidis, 1985a, 1990; Pinkel et al., 1988; Schardin et al., 1985). Since the 1990s, FISH combined with either epifluorescence microscopy (EFM) or confocal laser scanning microscopy (CLSM) became the favorite approach for studies of higher order arrangements of CTs as well as individual genes (see below).

### 3. Models of Nuclear Architecture and Experimental Evidence

#### 3.1. Current models

The severe limitations in our present knowledge of nuclear architecture become obvious, when we consider current models in recent scientific publications and textbooks (Fig. 1.2) (Cremer and Cremer, 2006b). Presently discussed concepts of higher order chromatin organization differ with regard to the fractions of the nuclear space occupied by transcriptionally permissive euchromatin and transcriptionally silent facultative heterochromatin, as well as the size distribution (DNA content) and
Figure 1. Different models of chromatin organization and topology of gene expression. (A) The chromosome territory–interchromatin compartment (CT–IC) model (Cremer and Cremer, 2001, 2006b; Cremer et al., 2000) argues for the coexistence of highly folded CTs built up from chromatin domains and a nearly DNA-free IC expanding between these domains. The perichromatin region contains decondensed chromatin and provides the border zone between the rather compact interior of chromatin domains and the IC (Fakan, 2004). The PK is the major functional, nuclear subcompartment. DNA transcription, RNA splicing, as well as DNA replication and DNA repair take place within...
compaction levels of chromatin loops/fibres. The CT–IC (chromosome territory–interchromatin compartment) model argues that nuclei are built up from two principal components, CTs and the IC (Cremer and Cremer, 2001, 2010; Cremer et al., 2000). The territorial organization of interphase chromosomes is now generally accepted as a basic principle of nuclear organization in both animals (Cremer and Cremer, 2001) and plants (Berr et al., 2006; Pecinka et al., 2004; Shaw et al., 2002) and may even hold for single cell eukaryotes, such as budding and fission yeast (Bystricky et al., 2005; Molnar and Kleckner, 2008). In multicellular organisms studied to date, a large chromatin fraction appears to be organized as ~1 Mb chromatin domains first detected in S-phase nuclei as replication foci, but later shown to be persistent higher order chromatin structures (Albiez et al., 2006; Jackson and Pombo, 1998; Visser and Aten, 1999). Larger chromatin clumps may be composed of clusters of ~1 Mb domains. According to the CT–IC model, ~1 Mb chromatin domains constitute a basic structure of CTs (Albiez et al., 2006; Berezney et al., 2005), but neither their ultrastructural organization nor the packaging of chromatin connecting these domains has been clarified to date (see below). The IC concept asserts an apparently DNA-free, contiguous space, which expands between the nuclear pores and the higher order chromatin network described above. The IC harbors nonchromatin nuclear domains such as interchromatin granules or splicing speckles as well as a variety of nuclear bodies (Albiez et al., 2006; Fakan, 2004; Verschure et al., 1999; Visser et al., 2000). The entire IC is separated from the more condensed interior of chromatin domains and/or higher order chromatin fibers by a layer of more decondensed chromatin and fibrogranular RNP constituents, termed the perichromatin region (PR) (Fakan and van Driel, 2007). Topographically, the PR, therefore, represents the utmost periphery of a given chromatin domain (cf. Figs. 1.4 and 1.8). (B) The interchromatin network (ICN) model (Branco and Pombo, 2006; reproduced with permission) proposes that euchromatin is made up from chromatin fibers, which intermingle more or less homogeneously by constrained diffusion both in the interior of CTs and between neighboring CTs. Blue dots signify interchromosomal contacts maintained by tethering. (C) The giant loop field (GLF) model (Chubb and Bickmore, 2003) suggests that transcription occurs on giant chromatin loops which expand from the surface of CTs and form a field of intermingling loops. When transcription ceases, the giant loops collapse back onto condensed core domain of CTs, which is typically visualized by chromosome painting. (D) The long-range interaction (LRI) model (Figure 4-66/page 241; Alberts et al., 2008, reproduced with permission) shows the most extreme version of a giant loop model. Giant loops can be very long and expand throughout the whole nuclear space in order to carry genes located on them even to very remote nuclear sites. In this way several genes can congress within the same “nuclear neighborhood for gene expression” or expression hub (Kosak and Groudine, 2004) and be transcribed there in a coregulated manner. In addition, the LRI model suggests genes on very long giant loops can reach distant nuclear neighborhoods for gene silencing. (See Color Insert.)
domain. Functionally, we argue that it presents the essential subnuclear compartment for DNA replication and repair, transcription, and pre-mRNA splicing (Fig. 1.2A).

If one wishes to define the interchromatin space simply as any chromatin-free nuclear space, such a space is an implicit part of any model of higher order chromatin arrangements including the proposition that the nucleoplasm is filled by intermingling chromatin fibers. The “lattice” model of interphase chromatin proposed by Dehghani et al. (2005) suggests a lattice-like network of 10 and 30 nm fibers. This structure yields a porous organization of chromatin with fibers intermingling at the borders of neighboring CTs. In agreement with this claim, the interchromatin network (ICN) model (Branco and Pombo, 2006) predicts that chromatin fibers and loops intermingle in a rather uniform way, both in the interior of individual CTs and between differentially labeled neighboring CTs, making any distinction between the interior or periphery of distinct chromatin domains functionally meaningless (Fig. 1.2B). In this ICN, loops may expand from one CT to meet loops from another CT. The ICN model does not provide any argument for a special nuclear topography of transcriptionally active and silent compartments.

Still, other models favor giant chromatin loops, which emanate from dense chromatin domains (Chubb and Bickmore, 2003; Fraser and Bickmore, 2007). The Bickmore group has argued that “the organization of chromosomes within the nucleus is probably somewhere in between the complete decondensation of chromatin fibers like spaghetti on a plate suggested 30 years ago and the model of a discrete territorial organization favored recently” (Mahy et al., 2002a) (Fig. 1.2C). For a short synonym in the following text, we name this model the GLF (giant loop field) model.

An extreme case of a giant loop model was proposed in the fifth edition of the textbook Molecular Biology of the Cell (Alberts et al., 2008) (Fig. 1.2D). According to this model, which for brevity, we will refer to as the LRI (long-range interaction) model, giant loops expand throughout the nuclear space in order to carry genes located on them even to very remote sites across the whole nuclear space. Several genes can congress within the same “nuclear neighborhood for gene expression” or expression hub (Kosak and Groudine, 2004) and be transcribed there in a coregulated manner. In addition, this model suggests nuclear neighborhoods for gene silencing in order to carry genes to such repressive nuclear compartments.

The lack of quantitative rigor has limited the usefulness of the models described above. To overcome this situation, chromatin polymer models have been developed, which make experimentally testable, quantitative predictions about functionally important features of the nuclear architecture, such as the expected size distribution of chromatin loops and chromatin compaction levels. Attempts have been made to explain the structure and nuclear arrangements of chromatin by random walk polymer models assuming a looped chromatin organization. The size of the loops predicted by different models
ranges widely. The random-walk/giant-loop model has argued for a random-walk backbone of an ill-defined nature to which chromatin loops of 3 Mb are attached (van den Engh et al., 1992). The multiloop-subcompartment (MLS) model has proposed rosette-like structures consisting of multiple 120-kb loops (Munkel and Langowski, 1998; Munkel et al., 1999), which provide a possible explanation for the organization of replication foci/∼1 Mb chromatin domains (Cremer and Cremer, 2001). A recent chromatin polymer model has assumed a broad range of loop sizes (Mateos-Langerak et al., 2009). All chromatin polymer models need to explain that chromatin loops representing an individual chromosome are spatially constrained to a confined nuclear subvolume, the CT.

3.2. Experimental evidence

3.2.1. Chromosome territories and interchromatin spaces

Whereas CTs are now fully accepted as a basic feature of nuclear organization, the question of an interchromatin space or more specifically of an IC as predicted by the CT–IC model (see above; Cremer and Cremer, 2001, 2010; Cremer et al., 2000) has remained controversial. An interchromatin space was first described in EM studies in the 1960s (Monneron and Bernhard 1969). After the (re)discovery of CTs the existence of a network-like space, called the interchromosomal domain (ICD), was suggested expanding mainly around CTs with little penetration into the CT interior (Cremer et al., 1993; Zirbel et al., 1993). Supposedly, genes were preferentially transcribed in a region of decondensed chromatin delineating CT surfaces and RNA transcripts were directly released in the ICD compartment. This concept was later supported by a series of studies from the Lichter group (Bridger et al., 1998, 2005; Gorisch et al., 2003, 2005; Reichenzeller et al., 2000; Richter et al., 2005). Accumulating evidence for genes transcribed both outside and in the interior of CTs (Mahy et al., 2002b; Verschure et al., 1999) is consistent with electron microscopic evidence for a network-like DNA-free space both outside and inside CTs (Visser et al., 2000). Notably, the conventional staining of ultrathin sections with uranyl acetate and lead citrate is unspecific. Accordingly, DNA, RNA, and proteins cannot be discriminate. Thus, it can be difficult, if not impossible, to distinguish a rather DNA-free IC full with RNPs and protein complexes from neighboring chromatin clusters. To do so it is necessary to combine EM studies with DNA-specific staining procedures. Indeed, such EM studies have provided strong evidence in favor of an interchromatin space which expands between chromatin clusters. Figure 1.3 shows the interchromatin space and the DNA distribution in a 2D micrograph and 3D EM reconstruction of a rat hepatocyte nucleus following specific DNA staining (Rouquette et al., 2009) (cf. Fig. 1.4). This 3D reconstruction is in full agreement with expectation of the CT–IC model (Fig. 1.2A) but not with
the ICN model (Fig. 1.2B). It was based on 170 sequential images obtained with a scanning electron microscope. After each image a slice of 50 nm was removed with a built-in ultramicrotome (Denk and Horstmann, 2004). The interchromatin space in rat hepatocyte nuclei occupied about 65% of the total nuclear volume, whereas endothelial cell nuclei in the same tissue occupied only about 40%. These values agree with 3D EM reconstructions of serial ultrathin sections of interphase nuclei indicating about half of nuclear space filled by chromatin (Esquivel et al., 1989; Lopez-Velazquez et al., 1996).

It should be noted that values for the relative space taken by chromatin and the interchromatin space, respectively, do not suffice to distinguish between the CT–IC and the ICN models, although the actual chromatin distribution predicted by both models is decisively different (Fig. 1.2A and B).

Based on this evidence, the hypothetical CT structure suggested by the CT–IC model may best be compared to a sponge of chromatin permeated by intraterritorial IC channels (cf. also Fig. 3 in Visser et al., 2000), as well as 3D reconstructions of CTs based on light optical serial sections (cf. Fig. 24 in Cremer and Cremer, 2006b). Individual CTs form an interconnected network of compact higher order chromatin domains and are closely associated with a second contiguous 3D spatial network observed both at the FM and
EM levels and called the IC or interchromatin space (Albiez et al., 2006; Visser et al., 2000).

EM studies demonstrating chromatin clusters can be integrated with other EM studies arguing for the packaging of a large proportion of mammalian chromatin into 60–130 nm “chromonema” fibers (Belmont and Bruce, 1994). In spite of circumstantial evidence for a nonrandom, dynamic organization of CTs described below, studies to define cell type- and species-specific differences between ultrastructural features of CTs are still in their beginning (Rego et al., 2008).

In stark contrast to the CT–IC model, the ICN model argues for high levels of intermingling between chromatin loops inside CTs and between CTs. Whereas evidence that space between chromatin adds up to about half of the total nuclear space fits with both the CT–IC model and the ICN model, one should be aware of the major differences of chromatin organization predicted by both models. The ICN model was proposed on the basis of FISH experiments with chromosome painting probes to thick cryosections (140–180 nm) from cell nuclei; the hybridized sections were first examined by FM and subsequently by TEM employing a detection scheme, where the hybridization sites of two differentially labeled chromosome paint probes were recognized with colloidal gold particles of different size. Occasional intermingling of neighboring CTs is consistent with a previously reported observation on in vivo labeled cells describing direct contacts between a labeled chromatin domain/territory and its unlabeled neighbor (Visser et al., 2000). In control experiments, Branco and Pombo (2006) performed EM on nuclei where histone H2B was indirectly immunolabeled with gold particles, and the positions of gold grains did not significantly change when cryosections were studied before and after mock FISH. Examination of the published electron micrographs does not, however, allow a morphological orientation in the section with regard to chromatin domains and other nuclear structures. Accordingly, the level of structural preservation in these cryosections remains doubtful. Nevertheless, one has an impression of a chromatin-poor/-free interchromatin space, which expands between chromatin domains (see Fig. 1J and K in Branco and Pombo, 2006). In order to resolve conflicting opinions about intermingling chromatin loops, it is necessary to provide quantitative data about the numbers, length and compaction levels of such fibers, and their 3D distribution.

3.2.2. Chromatin loops: How many, how long, and how compacted?
Although numerous studies have presented evidence for ~11 and ~30-nm-thick chromatin fibers as well as higher order chromatin configurations (Belmont et al., 1999; Gilbert et al., 2004, 2005), chromatin configurations above the ~11-nm fiber have remained controversial (Sapojnikova et al., 2009). Recent cryoelectron microscopic studies carried out on vitrified sections of in situ interphase nuclei of different cultured
mammalian cells (Bouchet-Marquis et al., 2006) (Fig. 1.4) or HeLa cell mitotic chromosomes (Eltsov et al., 2008) were unable to reveal 30-nm chromatin fibers. The variation in length of fibers/loops of different compaction levels and the fraction of the genome packaged in this way may strongly vary with the global transcription level of a given cell at different functional states. Such data are still lacking for most cell types.

Heitz (1928) originally introduced the terms heterochromatin and euchromatin to discriminate between genetically inactive and active chromatin. While constitutive heterochromatin typically lacks genes and is ubiquitously present as compact chromatin in all cell types of an organism, such as pericentromeric heterochromatin, the structural and/or functional definitions of facultative heterochromatin and euchromatin have remained ambiguous to date. The rapidly evolving field of epigenetics has provided for the first time possibilities to characterize functionally different types of chromatin at the molecular level (Berger, 2007; Brink et al., 2006; Trojer and Reinberg, 2007). Facultative heterochromatin comprises silent genes, which are inactive only under specific circumstances. The inactive X-chromosome in female somatic cells of mammals provides
a case in point. Arguably, facultative heterochromatin has a “closed” configuration in contrast to the “open” configuration of euchromatin. An “open” chromatin context, however, does not imply that all genes present in such a configuration are actually transcribed. Structural differences of active and inactive chromatin in the living cell are still surprisingly ill-defined. Transcriptionally poised or active chromatin may show a wide range of different compaction states. Thus, the notion of compact chromatin is not necessarily equivalent with the notion of heterochromatin. Despite this confusion there is an agreement that the relative amounts of active and inactive chromatin are developmentally regulated and can differ strongly in different cell types and different functional contexts. Whereas nuclei with a low transcription level, such as those in nonstimulated lymphocytes of the peripheral blood, typically show large blocks of condensed chromatin, nuclei with a high transcription rate, such as nuclei in PHA-stimulated lymphocytes, often exhibit highly fragmented condensed chromatin domains (Pompidou et al., 1984).

Several groups reported chromatin loops carrying specific clusters of genes expanding up to several micrometers away from the surface of their home CTs (Mahy et al., 2002a; Ragoczy et al., 2003; Volpi et al., 2000; Williams et al., 2002). Such an extrusion of a gene locus from a CT is not necessarily indicative of transcriptional activity, but also can reflect a poised state for activation (Ragoczy et al., 2003). Notably, the compaction level of one such giant loop studied in detail was about one order of magnitude higher than that of an extended 30-nm-thick chromatin loop (Albiez et al., 2006) (Fig. 1.5). Evidence for large numbers of giant loops expanding through major parts of the nuclear space as suggested by the LRI model (Fig. 1.2D) is meager. As a caveat, one needs to take into account that the sensitivity of chromosome painting experiments does not suffice to detect giant loops. 3D FISH experiments can only detect such loops and their true extension in the nuclear space with contig probes delineating the entire loop in question (Fig. 1.5). Both live-cell experiments and studies of fixed cell nuclei with TEM have supported the concept that most chromatin is compacted as focal chromatin domains with compaction levels above those of extended 30-nm chromatin fibers (Albiez et al., 2006). In live-cell experiments it was further demonstrated that the structural reorganization of CTs into mitotic chromosomes depends on the increased local compaction of chromatin—not the retraction of many giant loops expanding through a major part of the nucleus (Walter et al., 2003b). Again the limited sensitivity of present methods may have prevented the detection of occasional giant loops. Despite a considerable amount of information on the arrangement of CTs, their chromatin organization is not yet understood at the ultrastructural level. The particularly compact state of mitotic chromosomes makes it also extremely difficult to unravel their higher order structure (Belmont, 2006; Falconi et al., 2006; Wanner and Formanek,
At present we do not know in sufficient detail the structural transformation of CTs into mitotic chromosomes. Evidence that the positions of genes harbored by a CT can change during activation or silencing of genes raises the question whether functionally relevant, positional information provided by a specific topography of genes in a given CT is still reflected by the 3D organization of the respective mitotic chromosome or whether such information is lost during mitosis and thus must be fully restored during the next interphase, most likely during early G1.

**Figure 1.5** 3D-FISH of 15 BACs spanning a gene-dense region on 11p15.5 (cf. Fig. 1.2C). Human fibroblast nucleus (stained with TOPRO-3; false colored in gray) after multicolor 3D FISH of the two HSA 11 territories (green) together with a gene-dense 2.35 Mbs region from HSA 11p15.5. Fifteen BACs were used for the delineation of this region in false colors: red for the most telomeric, blue for the most centromeric BAC, and yellow for 13 BACs covering the intermediate region as a contig except for 350 kb in the middle (not shown). The nuclear shape is represented by a maximum Z projection of TOPRO-3 sections. The 3D reconstruction of the gene-dense region reveals a finger-like chromatin protrusion with a compaction factor of ~1:300 expanding from CT 11 (cf. Fig. 1 in Albiez et al., 2006). (See Color Insert.)
3.2.3. Nonrandom arrangements of chromosome territories and chromatin domains

Two cases of nonrandom higher order arrangements of CTs and subchromosomal regions have to be distinguished. Nonrandom radial arrangements describe the preferred location of specific chromatin structures, such as CTs, chromosomal subregions and genes, with respect to their 3D distance from the nuclear center or from the nuclear envelope. Nonrandom neighborhood arrangements reflect the proximity/clustering of such structures to an extent that cannot be explained as a consequence of a nonrandom radial organization.

A search for molecular mechanism(s) involved in dynamic changes of higher order chromatin arrangements must be based on a well documented and fully reliable descriptive analysis of these changes. During evolution nonrandom proximity patterns of CTs, specific chromatin segments or genes may have been established primarily for nonfunctional reasons such as geometrical constraints. Later evolution, the old tinkerer, to use Francois Jacob’s famous expression, may have exploited such opportunities leading step by step to a preference of functionally advantageous proximity patterns between distinct chromatin domains and other nuclear structures.

In most mammalian cell types studied to date, nonrandom radial CT positions were correlated with gene density and to some extent with chromosome size. In spherical cell nuclei, such as lymphocyte nuclei, positions of gene-poor CTs were typically found closer to the nuclear envelope than the positions of gene-dense CTs (Cremer et al., 2001, 2003; Croft et al., 1999). In flat ellipsoidal nuclei, such as nuclei in cultured fibroblasts, a chromosome size correlated distribution was found as the predominant feature (Cremer et al., 2001; Sun et al., 2000) although gene density-dependent features of CT positioning were also detected (Bolzer et al., 2005; Neusser et al., 2007). Notably, a study of bovine embryos generated by in vitro fertilization (IVF) revealed that this positioning is not present in pronuclei and blastomere nuclei during the first cell cycles but takes place only during major genome activation (Koehler et al., 2009). In murine cells lacking full-length lamin B1 or defective in processing its CAAX anchor positions of gene-dense and gene-poor CTs are affected (Malhas et al., 2007). In case of CTs, which are built from gene-poor and gene-dense segments, the latter were typically observed in a more interior position (Neusser et al., 2007). Across a wide evolutionary spectrum, ranging from primates and other mammalian cells (Bolzer et al., 2005; Grasser et al., 2008; Kupper et al., 2007; Mayer et al., 2005; Tanabe et al., 2002) to birds (Habermann et al., 2001), hydra (Alexandrova et al., 2003), plants (Mayr et al., 2003), and single cell eukaryotes (Postberg et al., 2005), a considerable amount of dense chromatin/heterochromatin is situated at the nuclear periphery, while other dense chromatin/heterochromatin surrounds the nucleoli as well as the internal nuclear regions outside nucleoli.
In contrast, euchromatin expands toward the nuclear interior together with dispersed interior clumps of condensed chromatin/heterochromatin. We refer to this organization as the conventional type of nuclear architecture. Possible functional reasons for this conventional architecture are unknown. A recent study of the mammalian retina (Solovei et al., 2009) has demonstrated that the nuclear architecture of rod cell nuclei is inverted in mammals adapted to low light conditions, while rod cell nuclei of mammals adapted to a diurnal life style possess the conventional architecture. In nuclei with the inverted architecture, all heterochromatin locates in the nuclear interior, while all euchromatin is located at the nuclear periphery. The inverted pattern forms by remodeling of the conventional one during postmitotic, terminal differentiation of rods. So far, the inverted nuclear architecture of rod cell nuclei from diurnal retinas seems to be a unique finding, indicating an adaptation of this nuclear architecture to the biophysical requirements of an effective channeling of photons toward the photoreceptors. The conventional architecture of eukaryotic nuclei may have prevailed in most cells during evolution, because it provides opportunities for more flexible chromosome arrangements facilitating a cell typespecific positional regulation of nuclear functions (Solovei et al., 2009).

Nonrandom neighborhood arrangements or proximity patterns between certain nonhomologous CTs were also discovered (Parada et al., 2002; Roix et al., 2003). This proximity, however, was typically detected in a minority of nuclei, but never in all nuclei of a given cell population (Parada et al., 2004). This finding argues either for the possibility that nonrandom CT neighborhood arrangements are functionally required only in a subset of nuclei (Parada et al., 2003) or for the transient nature of a given proximity pattern established only at certain periods during the cell cycle or at certain stages of postmitotic cell differentiation. Importantly, preferred CT arrangements seem to be probabilistic, that is, these arrangements occur more often than expected in case of a purely random arrangement, but they are not deterministic in the sense that all nuclei in a well-defined population of cells would reveal exactly the same arrangement (Zeitz et al., 2009). The analysis of a fixed cell population by chromosome painting resembles a snapshot of a group of individuals. Additional information on the dynamic behavior of the individuals is required to decide whether certain individuals already met before or will meet after the snapshot was taken. This can either be achieved by taking many snapshots of fixed samples at different time point or preferably by the observation of living cells. Numerous studies demonstrated cell type-specific differences of centromere arrangements (Billia et al., 1992; Borden and Manuelidis, 1988; Brero et al., 2005; Hu et al., 2008; Manuelidis, 1985b; Martou and De Boni, 2000; Park and De Boni, 1992; Solovei et al., 2004, 2009). Still, little is known about whether proximity patterns of whole CTs may change as well in correlation with changing functional states. Much more evidence for permanent or transient CT proximity patterns will be obtained when...
systematic live-cell studies of CT movements become possible in cycling and postmitotic cell types present in tissues.

Another old and largely unsolved problem concerns the question of nonrandom associations between homologous CTs in somatic cell types. Such associations are a hallmark of somatic cell nuclei of *D. melanogaster* and other Diptera species (Fritsch et al., 2006; Hiraoka et al., 1993; Lowenstein et al., 2004), whereas in cell types of many other species the spatial order of homologous CTs appears quite variable (Bolzer et al., 2005; Cremer et al., 1982a, 1982b; Pecinka et al., 2004; Scherthan et al., 1996). This issue is, however, complicated by the possibility that somatic homologous associations or pairing may be restricted to certain chromosomes in certain cell types and functional states. A study from Arnoldus et al. (1989) provides a point in case. These authors performed *in situ* hybridization experiments with normal human brain tissues using one probe specific for the 1q12 heterochromatic block of HSA 1 and another for the centromere of HSA 7. In the cerebral and the cerebellar samples they found two HSA 7 centromere spots in 82% and 83%, respectively, of the nuclei. *In situ* hybridization with the chromosome 1 probe showed only one large spot in 82% of the cerebellum nuclei, yet two smaller spots in 69% of the cerebral nuclei. Subsequent studies reported examples for homologous associations of centromeric domains in normal and tumor cell nuclei (Henikoff, 1997; Iourov et al., 2006; Koeman et al., 2008; Vadakkan et al., 2006). In contrast to somatic pairing in Diptera, somatic associations were only found in a minority of the evaluated nuclear samples. Accordingly, such associations may either occur transiently or may be entirely lacking in most nuclei. In any case, compelling evidence for functional implications is lacking. Based on a study on coordinate gene regulation during mouse hematopoiesis (Kosak et al., 2007) suggested that proximity in the form of chromosomal gene distribution and homolog association may be the basis for organizing the genome for coordinate gene regulation during cellular differentiation. As a caveat, we note that CTs preferentially located close to the nuclear center show significantly smaller 3D distances and a higher frequency of homologous associations as compared to CTs located at the nuclear periphery.

### 3.2.4. Mobility of chromosome territories and chromatin domains

Positional changes of CTs and chromatin domains as well as genes indicate the dynamic nature of higher order chromatin arrangements. Early experiments were performed with cells fixed at different times of development or after exposure to certain stimuli (De Boni, 1994). In a seminal investigation, Barr and Bertram (1949, 1951) described that upon electric stimulation of cat motor neurons, a “nucleolar satellite” (now known as the Barr body) moved from its usual position adjacent to the nucleolus, toward the nuclear membrane within a time course of several days. Borden and Manuelidis (1988) demonstrated a pronounced repositioning of the human X-territory
in neurons of both males and females in electrophysiologically defined seizure foci, whereas other CTs (HSA 1, 9, and Y) showed more subtle positional changes. De Boni and coworkers reported the rearrangement of centromeric satellite DNA in hippocampal neurons exhibiting long-term potentiation (Billia et al., 1992) and in mouse dorsal root ganglion neurons exposed to the neurotransmitter GABA (Holowacz and De Boni, 1991).

Live-cell experiments performed with cultured mammalian and *Drosophila* cells demonstrated locally constrained movements of subchromosomal domains (Abney et al., 1997; Bornfleth et al., 1999; Edelmann et al., 2001; Marshall et al., 1997). In HeLa cell nuclei, movements of chromatin domains appeared more pronounced during early G1 compared with the more constrained movements observed during G1 to late G2 (Edelmann et al., 2001; Walter et al., 2003b). Once established in early G1, the positioning of entire CTs appears stable for the whole interphase (Walter et al., 2003b; Zink et al., 1998). More studies are needed to distinguish chromatin movements, which are the result of random diffusion, from energy-dependent directed movements (Chuang and Belmont, 2007; Chuang et al., 2006; Levi et al., 2005). This question is particularly important with respect to reports of long-range chromatin movements involved in the congression of coregulated genes. Elegant studies with living budding yeast cells provided insights into the long-range compaction and flexibility of native chromatin (Bystricky et al., 2004).

The question, to what extent a given proximity pattern established between CTs during a given cell cycle may be transmitted to the next one, has remained controversial (Gerlich et al., 2003; Kalmarova et al., 2008; Thomson et al., 2004; Walter et al., 2003b). Chromosome movements during prometaphase required to establish the metaphase plate can lead to major changes of side-by-side chromosome arrangements in the metaphase plate compared with the side-by-side arrangements of the respective CTs during the preceding interphase (H. Strickfaden, A. Zunhamer, D. Koehler, and T. Cremer, unpublished data). In summary, available evidence argues (a) for a pronounced cell-to-cell variation of CT neighborhood arrangements in cell types studied so far, (b) for the stability of a given CT neighborhood arrangement once established at the onset of interphase until the next prophase, and (c) for major, probabilistic changes of chromosomes during mitosis (Cvackova et al., 2009; Walter et al., 2003b). As a caveat it must be emphasized that this evidence was obtained from a few cell types cultured in vitro.

### 3.2.5. Nonrandom arrangements, repositioning, and nuclear convergence of genes

Parameters correlated with nonrandom radial nuclear arrangements of chromosomal subregions and genes include regional gene density, transcriptional activity, and replication timing (Amrichova et al., 2003; Goetze et al.,
CORRECTED

PR

2007; Grasser et al., 2008; Hepperger et al., 2008; Kupper et al., 2007; Mayer et al., 2005; Murmann et al., 2005; Neusser et al., 2007; Sadoni et al., 1999). Since gene density, transcriptional activity, and replication time of a given chromatin segment are correlated with each other (Caron et al., 2001), it is not easy to distinguish between the relative contributions of these parameters to radial gene positioning. Several parameters may have an influence and it seems necessary to examine this problem on a gene-by-gene basis rather than making global statements. Notably, radial positions of a given gene can change significantly between cell types, excluding the possibility that gene positioning is determined solely by the DNA sequence environment in which a given gene is embedded (Hepperger et al., 2008).

Gene repositioning has emerged as an additional level of epigenetic gene regulation (Bartova and Kozubek, 2006; Lanctot et al., 2007a). During repositioning, genes move from a repressive nuclear environment into a neighboring compartment favorable for transcription or vice versa. For some genes their transcriptional silent or active state could be correlated with their positioning close to a domain of constitutive heterochromatin or away from it (Brown et al., 1999). In other cases the silent gene was positioned close to the nuclear envelope and the active one remote from it (Kosak et al., 2002). In multipotent progenitors and derived cell types from an in vitro model of murine hematopoiesis, Kosak et al. (2007) noted a significant clustering of coregulated genes, both with respect to their linear arrangement along mitotic chromosomes and the 3D arrangement of active genes in the nuclear interior.

The prevalence of heterochromatin localized at the lamina and the observation of silenced genes in this peripheral nuclear subcompartment has supported the concept that the nuclear periphery is a largely repressive environment for transcription (Schneider and Grosschedl, 2007). Recent studies, however, support a more complex picture (Akhtar and Gasser, 2007; Deniaud and Bickmore, 2009; Taddei et al., 2006; Towbin et al., 2009). Several groups succeeded to tether specific chromatin segments to the nuclear envelope in living cells. They found that some genes were suppressed when closely associated with the envelope, but that others were not (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008). Notably, A- and B-type lamins are organized into separate, but interacting, microdomains in the lamina and may contribute to form different microenvironments for gene regulation (Shimi et al., 2008). In a genome-wide survey of D. melanogaster Kc cells, van Steensel and coworkers detected ∼500 transcriptionally silent, late replicating genes that interact with B-type lamin (Pickersgill et al., 2006). Studies in yeast have shown the association of active genes with the nuclear pore complex (NPC) (Taddei, 2007). Considering the possible role of gene repositioning for gene regulation, one should not forget the importance of local fluctuations of unfolding chromatin with respect to the availability of genes to transcription machineries (Sato et al., 2004).
Of particular interest are hints that a long-range spatial nuclear convergence of genes, which are located many megabases apart on the same chromosome or even on different chromosomes, might be involved in mechanisms of gene activation or silencing (Bartkuhn and Renkawitz, 2008; Zuckerkandl and Cavalli, 2007). This phenomenon has been referred to as “gene kissing” (Cavalli, 2007; Kioussis, 2005). As an early example, LaSalle and Lalande (1996) presented 3D FISH evidence for the transient spatial association of the AS/PWS loci during late S phase. These loci comprise the genes involved in two intensively studied imprinting disorders, the Angelman syndrome and the Prader–Willi syndrome. The authors argued that transient “kissing” between the two loci is required for maintaining opposite imprints in cycling cells. This specific case of “kissing,” however, could not be confirmed in a later study (Teller et al., 2007). Since expression of Hox genes located on different chromosomes is precisely regulated and synchronized during development, 3D distances between these genes were compared in cryosections of developing mouse embryos (Lanctot et al., 2007b). While chromatin decondensation and nuclear reorganization of the HoxB locus was noted upon induction of transcription (Chambeyron and Bickmore, 2004; Chambeyron et al., 2005), the frequency of “kissing” events was not significantly different in cells expressing a high proportion of the Hox clusters when compared to cells expressing none or only a few Hox genes. These results indicate that coregulation of the different Hox clusters is not associated with colocalization of the loci at a single regulatory compartment.

In a 3D FISH study of Drosophila flies, Bantignies et al. (2003) demonstrated the involvement of Fab-7, a well-defined cellular memory module, in long-distance interactions in cis and trans. Since then 3C experiments, in part reconfirmed by 3D FISH experiments, demonstrated numerous “kissing events” between genes in cis and trans (Krueger and Osborne, 2006; Lomvardas et al., 2006; Osborne and Eskiw, 2008; Osborne et al., 2004, 2007; Spilianakis et al., 2005; Tiwari et al., 2008; Williams and Flavell, 2008; Zhao et al., 2006). Evidence obtained by these experiments showed a percentage of colocalization between “kissing” genes large enough, say ≥ 10%, to consider a random occurrence quite unlikely.

DNA interactions in cis abound a few megabases around a sequence chosen as a bait to search for interactions with other sequences (de Laat and Grosveld, 2007). In this case, constrained Brownian movements of chromatin domains are likely sufficient to enable a “kiss” (Bornfleth et al., 1999; Chuang and Belmont, 2007). Constrained Brownian movements may also suffice to reposition silent genes located in a repressive “heterochromatic” compartment into a neighboring transcriptionally favorable “euchromatic” compartment. Repositioning events demonstrated to date at light microscopic resolution may be just the tip of the “iceberg” of dynamic events taking place between decondensed chromatin in the PR and underlying
condensed chromatin at submicroscopic resolution. The local chromatin environment may finally turn out to be much more dynamic than previously thought.

More sophisticated multicolor 3D FISH and evaluation schemes are required to clarify the topography of these "kissing" events. As a case in point we consider an experiment with a sample of diploid cell nuclei, where two genes located on different CTs are visualized together with painting of the nonhomologous CTs in different colors. Such a scheme could show whether pairs of colocalized genes are typically located remote from these CTs on giant loops or whether colocalization requires direct contact between a given pair of CTs. The loops could be visualized simultaneously with contigs of BAC pools covering a few megabase pairs of the immediate neighborhood of these genes in a third color. In addition to the determination of the fraction of colocalized genes in an unbiased sample, it is important to measure also 3D distances between pairs of non-colocalized genes. Such measurements could reveal whether these pairs show a range of 3D distances compatible with the possibility of a "kissing" event brought about by constrained Brownian motions. In case that a very variable or even random distribution of 3D distances between non-colocalized pairs of genes would be discovered, we would assume that pairs of genes with large distances, for example, >5 μm, would be unlikely candidates for a transient "kiss" elicited by constrained Brownian motion.

A recent study of primary cultures of human mammary epithelial cells and the human breast cancer cell line MCF7 describes rapid 17-estradiol (E2)-induced interactions in \textit{trans} between the GREB1 and the TFF1 loci located on chromosomes 2 and 21, respectively (Hu et al., 2008). Notably, the authors emphasize that HSA 2 and 21 territories were independently localized before E2 treatment. A significantly increased frequency of TFF1:GREB1 interactions, however, could already be demonstrated 15 min after E2-treatment (about 20% vs. 5% at time 0) and this fraction increased to nearly 50% within 1 h. About half of the cells exhibited monoallelic interactions, the other half exhibited biallelic interactions in \textit{trans}. Although HSA 2 and 21 territories became intimately associated in many cells, the frequency of E2-induced TFF1:GREB1 interactions was even higher than HSA 2 and 21 territory associations, suggesting the contribution of long-distance DNA looping in addition to movements of the entire territories toward each other. Movements resulting in TFF1:GREB1 "kissing" could be prevented by treatment of the cells with latrunculin, a drug that blocks actin polymerization, with Jasplakinolide that inhibits depolymerization of actin networks, by siRNA knockdown of nuclear Myosin-I and finally by microinjection of antibodies against Myosin-I or γ-actin. Finally, Hu et al. (2008) noted that upon E2 treatment the interacting TFF1/GREB1 loci became intimately associated with SC35-positive speckles (or interchromatin granules) and speculated that these granules may function as hubs for...
gene networking in the nucleus. This speculation contradicts evidence, which argues that interchromatin granules are a DNA-free domain located in the IC (Thiry, 1995b).

3.2.6. Local chromatin dynamics of transcription: Role of the perichromatin region

Like DNA replication, the nuclear topography of transcription sites was first investigated by methods of ultrastructural cytochemistry combined with EM autoradiography using tritiated uridine and later with immuno-EM and halogenated RNA precursors. Transcription sites have been demonstrated to occur, after labeling pulses as short as 2 min, predominantly in the PR (Fakan and Bernhard, 1971). This region is also the nuclear subcompartment where most factors involved in pre-mRNA/hnRNA formation accumulate, such as RNA Pol II, PolyA polymerase, and different other RNA processing factors (Cmarko et al., 1999; Fakan et al., 1984; Spector et al., 1991; Trentani et al., 2003) as well as DNA–RNA hybrids (Trentani et al., 2003). A special contrasting method for ultrathin sections allowed the visualization of ribonucleoprotein constituents in the PR, called perichromatin fibrils (Bernhard, 1969; Monneron and Bernhard, 1969). A perichromatin fibril represents the in situ form of a nucleoplasmic RNA transcript (Fakan, 1994, 2004; Fakan et al., 1976; Nash et al., 1975; Puvion and Puvion-Dutilleul, 1996) and also the site where cotranscriptional splicing obviously occurs (Biggiogera et al., 2008; Fakan, 2004).

The predicted interplay between chromatin topography, chromatin dynamics, DNA transcription and replication differs starkly between the CT–IC model, and the ICN and GLF models. According to the CT–IC model, one expects the localization of active genes within the PR, while silent genes might be located in the condensed interior of chromatin domains. The alternative that silent genes are also located at the periphery of chromatin domains is worth considering. This hypothesis has gained support by a study performed with two different human cell lines and with rat liver tissue showing the preferential location of Polycomb group (PcG) proteins HPC2, HPH1, BMI1, and RING1 in the PR, whereas they were virtually absent from the interior of condensed chromatin (Cmarko et al., 2003). Despite the substantial evidence provided in this review in favor for the existence of the IC and the PR as two topographically neighboring and functionally interacting, yet structurally distinct nuclear compartments, this concept has not been taken into account in pertinent, recent reviews (Fedorova and Zink, 2008, 2009; Fraser and Bickmore, 2007; Göndör and Ohlsson, 2009; Nunez et al., 2009; Pai and Engelke, 2010; Pombo and Branco, 2007; Sexton et al., 2009; Sutherland and Bickmore, 2009).

Improvements of RNA FISH technology have provided the possibility to study gene expression at the single cell level with high spatial and temporal
resolution (Levsky et al., 2002). We restrict the following considerations to the dynamic topography of transcription (Sinha et al., 2008). Everybody agrees on one point: transcription caught in action is performed on a short piece of naked DNA which has entered a channel-like part of the transcribing RNA Pol II molecule (Kettenberger et al., 2003). Whether the transcription machinery moves along the DNA or whether the DNA is pulled through RNA Pol II within an immobile transcription machinery, or whether both possibilities may be realized in vivo, is not known.

3.2.7. Nuclear topography of transcription: Transcription factories
The concept of transcription factories (TFs) provides a different interpretation of transcriptionally active compartments (Jackson, 2005; Sexton et al., 2007; Sutherland and Bickmore, 2009). Immunocytochemistry using brominated RNA precursors combined with FM allowed to observe some 300–500 transcription sites as multiple tiny spots (Jackson et al., 1993; Wansink et al., 1993), whereas Iborra et al. (1996) reported a spotted appearance of some 2100 RNA Pol II sites per nucleus (range 2000–4400). Jackson et al. (1993) coined the term TF, assuming that these foci represent nuclear machineries, where transcripts are both synthesized and processed. Subsequent studies reported that RNA FISH signals from various transcriptionally active genes were typically (90% or more) associated with RNA Pol II domains or factories (Osborne et al., 2004, 2007; Ragoczy et al., 2006). Based on the assumption that the total number of genes transcribed at a given time is considerably higher than the number of detectable sites of ongoing transcription, it was proposed that several genes can be simultaneously transcribed by a single TF (Fraser and Bickmore, 2007). The problem of estimating the number of genes transcribed in a given cell at a given time point has become more complex due to the discovery of transcriptional pulsing, that is, the fact that individual genes are turned on and off at irregular intervals (Chubb et al., 2006). Furthermore, RNA FISH signals from genes sparsely transcribed away from TFs might not be distinguishable from diffuse fluorescent background.

In published nuclear images, TFs appear quite variable with regard to numbers, shapes, and sizes. It is not clear to what extent notable differences between images of TFs, provided by different studies, reflect specifics of different cell types or different methods of TF visualization (nascent RNA, RNA Pol II) and image recording. The size of TFs and the percentage found for associations between RNA FISH signals and RNA Pol II foci depends, of course, to some extent on the threshold used for signal segmentation. Yet, the discrepancies between FM studies describing large numbers of quite tiny spots (Iborra et al., 1996; Jackson et al., 1993) and others presenting smaller numbers of much larger TFs of irregular form and shape with diameters of several hundred nanometers up to the micrometer scale (Sexton et al., 2007) are conspicuous. It seems possible that TFs described by
different authors either as accumulations of newly synthesized RNA or RNA Pol II foci represent either different domains or different components of the same domains. In conclusion, although TFs have been taken for granted as distinct nuclear domains (Mitchell and Fraser, 2008), we still lack compelling ultrastructural, cytochemical, and molecular evidence for their existence as higher order functional units and for their topographical relationships with chromatin. Accordingly, concepts about their function as units for the coregulated, simultaneous transcription of several genes also remain speculative to date (Fig. 1.6A).

Similarly, presently available data do not provide compelling evidence for the nuclear topography of TFs. In the context of the ICN model (Fig. 1.2B), we would expect that TFs should be present wherever they are needed within the masses of intermingling chromatin loops, be it in the interior or periphery of intermingling CTs. The GLF model (Fig. 1.2C) argues for the preferential location of TFs within fields filled with giant loops expanding between core CTs and carrying transcriptionally active genes. When transcription ceases, these loops collapse back onto the condensed core of a CT (Chubb and Bickmore, 2003). Indeed, proponents of the TF concept have argued for the preferential location of these transcription machineries in the space between CTs filled with chromatin loops carrying active genes (Fig. 1.6B) (Fraser and Bickmore, 2007). In contrast, the CT–IC model proposes that sites of transcription are restricted to the PR with its width of about 100–200 nm (Fakan and van Driel, 2007) (Fig. 1.4). Accordingly, perichromatin fibrils still attached to DNA should be found everywhere within IC channels with a width < 400 nm, while in wider IC channels or lacunas, nascent RNA should not be significantly detectable in the interior. Numerous EM studies cited above support this assumption.

At the present state of knowledge, we cannot exclude that studies of a wider variety of cell types from different species, in particular cells with high demands of global transcriptional activity, may provide examples where many chromatin loops expand from compact chromatin domains beyond the typical 100–200 nm width of the PR. In the case that intensive comparative studies fail to provide such examples, the functional importance of the PR as the nuclear subcompartment for transcription would be further strengthened.

3.2.8. Routes of mRNA export

Whereas cotranscriptional splicing of nascent RNA present in perichromatin fibrils seems well documented, further possible modifications of these fibrils and routes to nuclear pores are still unsolved questions. Obviously, only a fraction of perichromatin fibrils contains RNA messages needed for protein synthesis in the cytoplasm, while others may contain RNAs which are retained and may serve regulatory processes in the cell nucleus. Many
Figure 1.6 Transcription factories. (A) Model of a transcription factory showing transcribed chromatin loops simultaneously moving through the factory at different sites (adapted by permission from Macmillan Publishers Ltd: Chakalova et al., 2005, copyright 2005). (B) Different types of gene interaction occurring in *cis* and *trans* in the interior of the interchromatin space (adapted by permission from Macmillan Publishers Ltd: Fraser and Bickmore, 2007, copyright 2007). The model argues that functionally important gene interaction can take place anywhere in this nuclear compartment. In contrast, the CT–IC model predicts that genes are localized within the PR (cf. Figs. 1.4 and 1.2A).
perichromatin fibrils detach from chromatin and move into the interchromatin space (Puvion and Moyne, 1978), whereas others may move within the PR toward the nuclear pores. In addition to perichromatin fibrils, perichromatin granules are typical RNA-containing nuclear constituents observed within the PR. Neither the formation nor the role of these granules has been clearly demonstrated to date. It has been shown that some perichromatin fibrils are able to form perichromatin granules, which may play a role in the storage/export of messenger RNA (Fakan, 2004; Vazquez-Nin et al., 1997).

Several groups analyzed Poly(A)RNA movements in the nucleus of living cells (Dirks and Tanke, 2006; Gorisch et al., 2005; Misteli, 2008; Politz et al., 2006). Shav-Tal et al. (2004) found that nucleoplasmic RNA moves by simple diffusion and in no-directional manner, while Zachar et al. (1993) suggested a channeled diffusion for intranuclear transport of pre-mRNA throughout the Drosophila polytene nucleus (Kramer et al., 1994). Poly(A)RNA moves equally well in speckles and in surrounding nucleoplasm (Politz et al., 2006) and the movement is not disturbed when transcription is blocked (Molenaar et al., 2004). In studies of U2OS cells, Shav-Tal et al. (2004) and Molenaar et al. (2004) found that diffusion coefficients of the predominant RNA fraction in the nucleus ranged from 0.01 to 0.09 μm²/s, whereas Politz et al. (2006) found a coefficient 5- to 10-fold larger in HeLa cells. In addition to this predominant fraction, an immobile or very slowly moving RNA population was reported (Molenaar et al., 2004; Politz et al., 2006; Shav-Tal et al., 2004). The question of whether RNA movements in the nucleus are energy-dependent or not has remained a controversial topic. Molenaar et al. (2004) claimed that RNA mobility requires energy, while Shav-Tal et al. (2004) and Politz et al. (2006) argued that it does not. When following single RNA molecules in living cells, Vargas et al. (2005) proposed that mRNP complexes moved by diffusion through the interchromatin space but needed energy to resume their motion after they became stalled.

3.2.9. Nuclear topography of DNA replication

The topography of DNA replication has been studied extensively by methods of ultrastructural cytochemistry combined with EM autoradiography and later with immunocytochemistry. Early EM studies showed that DNA replication does not require an association with the nuclear membrane (Fakan et al., 1972; Huberman et al., 1973; Ockey, 1972). EM studies using short pulses with labeled nucleotides revealed DNA replication sites in the PR regardless of the size of chromatin domains (Fakan and Hancock, 1974; Jaunin and Fakan, 2002). During pulse-chase experiments, newly synthesized DNA was rapidly relocated from the PR into the interior of chromatin clumps, suggesting a permanent movement of DNA between the periphery and interior of chromatin domains in the course of the
synthetic period (Jaunin et al., 2000). As in the case of transcription, we tend to assume a stepwise process of chromatin de- and recondensation. Small, decondensed segments of chromatin prone for replication expand into the PR, become replicated, and are subsequently reconfigured as a higher order structure. Although movements of replication machineries along DNA strands cannot be ruled out, it is also possible that replication machineries are fixed within the PR whereas DNA is moving. In HeLa cells (Hozak et al., 1994; Philimonenko et al., 2006), DNA replication was reported to take place in nuclear body-like structures called replication factories. Further studies with improved imaging methods are necessary to refine the dynamic topography of chromatin and essential machineries involved in both transcription and DNA replication, and to explore the structural implications of mechanisms that restore or modify chromatin organization after DNA replication (Corpet and Almouzni, 2009).

3.2.10. Nuclear topography of DNA repair

In contrast to the intensely studied biochemistry of DNA repair and factors involved in these processes (Brugmans et al., 2007; Caldecott, 2008; Feuerhahn and Egly, 2008; Hsieh and Yamane, 2008; Lieber, 2008; Peng and Karpen, 2008; Wyman and Kanaar, 2006), the nuclear topography of DNA repair (and possibly also of repair of epigenetic damage) have been explored much less to date (Aten et al., 2004; Falk et al., 2007; Folle, 2008; Krawczyk et al., 2008; Misteli and Soutoglou, 2009; Mortusewicz et al., 2005, 2006, 2008; Solimando et al., 2009).

Machineries for the repair of DNA may be built up directly at the nuclear sites where the damage is inflicted. Alternatively, it seems possible that repair machineries are first assembled at other sites and then moved to sites of damage or that damaged DNA/chromatin needs to be repositioned to a nuclear compartment favorable for the execution of repair. Recent evidence has supported the hypothesis that the PR—in addition to its roles in DNA replication and transcription—also serves as the preferential nuclear compartment for DNA repair. An electron microscopic investigation of nucleotide excision repair (NER) in human cell lines has recently shown that following UV-irradiation, XPA and XPC, two proteins involved in the chromatin-associated NER complex, accumulate within the PR. In contrast to XPA, significant amounts of XPC were also found in the compact interior of chromatin domains (Solimando et al., 2009). The authors speculate that both XPA and XPC may be essentially required to execute NER within the PR, but that XPC may play an additional role in the recognition of damaged DNA in the interior of compact chromatin domains. Accordingly, they hypothesize that damaged sites produced in the interior must first be moved to the PR. Notably, the resolution of conventional light microscopy is not sufficient to detect the relocation of damaged DNA (<250 nm) as suggested by Solimando et al. (2009). These new observations emphasize the importance
to study the topography and kinetics of DNA repair in space and time (4D) at the ultrastructural level. It is not yet known, whether the PR, similarly to its role in excision repair, presents the preferred nuclear compartment for DNA double strand break (DSB) repair.

Laser and ion microbeams have provided particularly useful tools to study the topography of DNA repair in single cells. Early laser–UV-microbeam studies ($\lambda = 257$ nm) demonstrated the possibility to induce targeted DNA-photolesions yielding excision repair restricted to CTs exposed at a microirradiated nuclear site. As expected, the induction of sister chromatid exchanges and chromosome aberrations was restricted to mitotic chromosomes bearing the damaged DNA (Cremer et al., 1982a,b; Raith et al., 1984; Zorn et al., 1979). Unexpectedly, however, posttreatment of microirradiated cells with caffeine (1–2 mM) synergistically enhanced chromosome damage yielding mitotic cells with “shattered” chromosomes (Zorn et al., 1976). Caffeine has long been known as an inhibitor of postreplication repair and more recently was found to inhibit the kinases ATM and/or ATR (Johansson et al., 2006). In some cases the “shattering” effect appeared restricted to mitotic chromosomes bearing the microirradiated chromatin (partial chromosome shattering, PCS). In other cases, however, the whole chromosome complement appeared “shattered” (generalized chromosome shattering, GCS). Apparently, chromosomal complements with PCS and GCS resulted from a failure of chromosome condensation rather than from extensive DNA fragmentation (Cremer and Cremer, 2006b). In UV-microbeam experiments, where a given incident energy was either concentrated to a small part of the nucleus or distributed over approximately the whole nuclear area, the fraction of mitotic cells with GCS obtained after posttreatment with caffeine increased with the total incident energy and was independent of the distribution of repair sites (Cremer et al., 1981). As an attempt to explain this unexpected phenomenon, a factor depletion model was proposed (Cremer and Cremer, 1986). It argues for a limited pool of certain, still unknown factors, which are recruited at sites of DNA repair, but also involved in the “maturation” of replicated chromatin necessary for a proper condensation process of mitotic chromosomes. GCS occurs under conditions where the available factor pool becomes exhausted.

More recently, laser-microbeam experiments were used to study the space–time organization of single and double strand DNA repair (SSBs and DSBs) in nuclei of living cells (Lukas et al., 2005). Prior to microirradiation, DNA was sensitized by the incorporation of halogenated thymidine analogs and in vivo staining with the dye Hoechst #33258. Microirradiation experiments of nuclear areas with focused laser light in the UVA range demonstrated the rapid formation of γ-H2AX foci as well as the accumulation of repair proteins, such as Nbs1 and Rad51, at nuclear sites carrying the microirradiated chromatin (Lukas et al., 2003, 2004; Tashiro et al., 2000;
Walter et al., 2003a). When cells were fixed 30–90 min after microirradiation of nuclei with letter-like patterns, Rad51 accumulations still revealed the form of these letters. This result argues against major movements of microirradiated chromatin during this period.

Focused X-rays (Folkard et al., 2001) or focused beams of energetic protons or heavy ions have also been used for the targeted irradiation of nuclei in living cells (Barberet et al., 2005; Folkard et al., 2001; Frankenberg et al., 2008; Greif et al., 2004; Hauptner et al., 2004; Heiss et al., 2006). A series of DNA single and double strand breaks (SSBs and DSBs) can be produced along the route of a given ion through the nucleus providing unique possibilities to study the assembly and disassembly of repair factors at sites of SSBs and DSBs, as well as the recruitment and subsequent release of repair factors involved in DSB repair. At sites of targeted SSBs and DSBs, the rapid formation of γ-H2AX was observed due to the phosphorylation of H2AX Ser 29 by the kinase ATM (Hauptner et al., 2004). Indirect immunocytochemistry on cells fixed at different time points after targeted ion-beam microirradiation at different nuclear sites demonstrated the accumulation of factors involved in DSB repair, such as 53BP1, Mdc1, and Rad51, within a few minutes after ion-microbeam radiation of selected nuclear sites. The patterns of accumulated repair factors induced by multiple targeted hits of single ions remained visible for many hours arguing against large-scale chromatin (Hauptner et al., 2006) (Fig. 1.7).

Figure 1.7 Microscopic observation of γ-H2AX accumulations following ion-microbeam irradiation. (A, B) Multiple ion-microbeam irradiation with single 100 MeV 16O ions was performed to generate stripes of damaged chromatin in nuclei of living HeLa cells with a distance of about 10 μm (Hauptner, 2006; published with permission of the author). Cells were fixed 30 min (A) and 15 h later (B). γ-H2AX generated by ATM-catalyzed phosphorylation of serine 139 of the histone variant H2AX was detected by indirect immunofluorescence with anti-γ-H2AX-specific antibodies. Notably, γ-H2AX positive stripes could still be detected 15 h after ion-microbeam irradiation with a similar geometry as in cells fixed after 30 min. Notably, the stripes detected after 30 min were still mostly parallel in different nuclei. In contrast, the stripes detected after 15 h revealed different directions in different nuclei indicating a rotation of nuclei (Hauptner et al., 2006).
The sequential generation of DSBs at selected nuclear sites with chosen time intervals was employed to study at the single cell level, whether earlier and later generated DSBs may compete for repair factors (Greubel et al., 2008a,b). Competition may be brought about by different on/off kinetics affecting the binding and release of different repair factors at DSBs, different pool sizes, synthesis and degradation of repair factors as well as a dynamic sequence of chromatin modifications at sites of DSBs. Tight binding of certain repair factors for prolonged times at sites of DSB repair generated earlier in a nucleus may result in the undersupply of such factors at additional sites of DSBs generated later. The experiments described above suggest such a competition effect between earlier generated and later generated DSBs for the repair factors 53BP1 and Rad51.

Most recently, transgenic human cell lines (HeLa, U2OS) were produced, which express one of the GFP-tagged DSB repair proteins Rad5, Rad52, 53BP1, and hMdc1. These cell lines were used to follow directly the recruitment of repair proteins to ion-microbeam irradiated nuclear sites with DSBs in the nuclei of single living cells (Hable et al., 2008). Visible recruitment of Mdc1-GFP to sites of DSB repair was already detectable 10–20 s following ion-microbeam irradiation. Mdc1 directly binds γ-H2AX (Stucki et al., 2005). In contrast, visible recruitment of Rad51-GFP and Rad52-GFP required 10–15 min. These preliminary results underline the potential of ion-microbeam studies to investigate the sequence and kinetics of the recruitment of repair factors.

4. Perspectives: Open Questions and Possibilities to Answer Them

4.1. Studies of higher order chromatin arrangements

Despite the evidence spelled out above for some evolutionary widely conserved features of nonrandom higher order chromatin arrangements, it is important to note the astounding variability of such arrangements both with regard to different cell types of a given organism (Hoffmann et al., 2007; Olins et al., 2008; Solovei et al., 2009) and species-specific peculiarities. In this context it is important to study nuclei in a wide range of eukaryotic species from distant parts of the evolutionary tree, including nuclei with strikingly different phenotypes, such as macronuclei of Ciliate species (Olins et al., 1981; Postberg et al., 2005, 2008), nuclei of dinoflagellates (Moreno Diaz de la Espina et al., 2005), nuclei in endocycling cells of the urochordate Oikopleura dioica (Costas and Goyanes, 2005; Spada et al., 2007), and nuclei of Trypanosoma brucei (Navarro et al., 2007).

It is important to be aware that evidence for a nonrandom spatial proximity of CTs (or chromosomal subregions down to individual genes)
does not prove that this proximity is required for a specific functional interaction. Deviations from a random arrangement may have simple and functionally irrelevant causes. For example, a different arrangement of large and small CTs may be brought about by different geometrical constraints acting in nuclei with different shapes (Bolzer et al., 2005). At this point it is not known whether mechanisms responsible for different nuclear shapes act from inside the nucleus and/or from outside. For example, the shape and compaction of CTs may affect the shape of the nucleus. Alternatively, the nuclear shape may be modified by interactions of the nuclear envelope with the cytoskeleton and even an extracellular matrix (Gieni and Hendzel, 2008). Understanding the functional implications of nuclear architecture and mechanisms necessary to achieve, maintain, and alter higher order chromatin arrangements during development and cell differentiation is still a far-off goal. Present knowledge about higher order chromatin structures and their arrangements in different cell types of a given organism and similarities, as well as differences of such arrangements in corresponding cell types of different species are still too fragmentary to distinguish evolutionarily shared principles, which may be valid in all eukaryotes, from cell type- and species-specific differences (Foster and Bridger, 2005).

4.2. Nuclear topography and models of the nuclear architecture

We need to integrate the level of nuclear architecture with the levels of epigenetics and of genome organization (Fig. 1.1) (Varga-Weisz and Becker, 2006; Wallace and Felsenfeld, 2007) and define the components essential for higher order chromatin organization and its changes due to internal and external stimuli (Cai et al., 2006; Carter et al., 2002; Galande et al., 2007). Possible implications of nuclear architecture for the ability of proteins to locate specific target sequences or structures among a vast excess of nonspecific DNA are not understood (Cremer et al., 1993; Gorman and Greene, 2008; Kampmann, 2005). We are still ignorant about the possible functional role(s) of an evolutionarily conserved radial distribution of gene-dense and gene-poor chromatin and cell type-specific proximity patterns, nor do we know the reasons for changes of chromatin arrangements in nuclei of cycling cells and during postmitotic terminal cell differentiation. We do not know whether changes of higher order chromatin arrangements are the cause or effect of functional changes or are even functionally meaningless, not to speak of the mechanism(s) which are necessary to accomplish such changes. One concept, which has obtained prominence during recent years, suggests expression hubs, where the localized position of multiple coregulated genes would facilitate their intercommunication by helping to form and then utilize localized concentration of regulatory
proteins (Kosak and Groudine, 2004). However, evidence for the structural nature of such hubs is lacking.

The different models of nuclear architecture discussed above should not necessarily be considered as mutually exclusive. They may rather accentuate different possibilities, which may be realized in some cell types but not in others. While the presence of CTs in nuclei of multicellular organisms has become a generally accepted feature of models of the nuclear architecture, the concept of a structurally and functionally distinct IC has remained controversial. This concept has been proposed as an essential part of the CT–IC model (Cremer and Cremer, 2001), but disputed by competing models such as the ICN and GLF models. Contrary to other models we argue that the IC and the PR are two structurally distinct and functionally interacting nuclear subcompartments, and that the PR serves as the nuclear subcompartment for transcription and RNA splicing, as well as DNA replication and according to most recent evidence also for repair (see above).

Figure 1.8 illustrates the contrasting views of the functional nuclear architecture proposed by the CT–IC model (Fig. 1.8A) as compared to a chromatin loop model arguing for numerous large chromatin loops with a thickness of $\sim 11$ and $\sim 30$ nm, respectively (Fig. 1.8E). According to the CT–IC model one would expect that snapshots of the chromatin organization of nuclei at any given time point should only reveal a relatively small fraction of chromatin present as extended $\sim 11$ and $\sim 30$ nm chromatin fibers or loops, whereas the ICN and GLF models argue for an extensive contribution. The space–time structure of $\sim 1$ Mb domains or clusters thereof (Fig. 1.8A) as well that of higher order chromatin fibers is unknown. The CT–IC model speculates that (a) each $\sim 1$ Mb domain is built up from a series of $\sim 100$ kb chromatin loop domains and (b) the smallest branches of the IC end within $\sim 1$ Mb chromatin domains (Cremer and Cremer, 2006b), but compelling experimental evidence is lacking. In particular, we do not know how much DNA is present in linearly arranged chromatin fibers of different compaction levels. With diameters of only a few hundred nanometers, the structure of $\sim 1$ Mb chromatin domains cannot be imaged by experiments using the resolution of conventional light microscopy. The CT–IC model takes into account perichromatin fibrils as in situ forms of single nascent hnRNA transcripts being produced in the PR. Aggregates of PFs may reflect the ongoing, independent transcription of several closely adjacent genes or multiple transcripts on the same gene. Such multiple transcripts were observed with EM on spread transcription complexes from different cell types (Foe et al., 1976; Harper and Puvion-Dutilleul, 1979; McKnight and Miller, 1979; Puvion-Dutilleul et al., 1978). The chromatin loop model takes into account TFs, which can transcribe several genes simultaneously. TFs are located within fields of intermingling chromatin loops, protruding from condensed chromatin domains. These different views of the nuclear topography of transcription cannot easily be
Figure 1.8 Schematic drawing comparing two models of transcription organization within the *in situ* nuclear landscape. (A) A scheme of a partial nuclear section according to the CT–IC model (cf. Fig. 1.2A) with ∼1 Mb chromatin domains and clusters of several such domains, as well as the interchromatin compartment expanding between them with splicing speckles and nuclear bodies. An enlargement of the boxed insert is shown in (B) and (C). (B–D) According to the CT–IC model, major transcriptional activity is expressed in the PR occurring on the border of chromatin domains (roughly delimited by the red dotted line). In the case where the chromatin domains (in gray) are close to each other (within a distance of about 400 nm or less), Brownian chromatin movement may shortly put transcription sites together (B and D, blue dotted line) and then separate them again (C). (E) This partial nuclear section differs from the situation presented in (A) by the assumption that the interchromatin compartment in addition to splicing speckles and nuclear bodies contains numerous chromatin loops expanding from higher order chromatin domains, as well as transcription factories. An enlargement of the boxed insert is shown in (F) and (G). Note that a perichromatin region (PR)
reconciled. Evidence that RNA Pol II is enriched in the PR (Cmarko et al., 1999) raises the question whether large TFs, seen after immunostaining of RNA Pol II (Sexton et al., 2007), could be reconciled with the perichromatin compartment model of transcription by the assumption that they represent a chromatin domain with RNA Pol II labeled in the PR surrounding the compact core of this domain; the fact that the whole domain shows fluorescent staining could simply be caused by the limited axial resolution. Smaller TFs may consist of aggregates of perichromatin fibrils. If so, we would expect that such TFs should be localized in the PR rather than in the middle of a field of intermingling chromatin loops. The TF hypothesis argues for a single structural unit, which serves several genes simultaneously. In the case of the chromatin loop model, genes being transcribed within a single TF will stay together despite Brownian chromatin motions (cf. Fig. 1.8F with G). It was reported that TFs visualized as RNA Pol II foci could be detected as persistent structures even in the absence of transcription (Mitchell and Fraser, 2008). In our view, a cluster of perichromatin fibrils may represent a transient spatial association without a functional necessity to keep this association for a prolonged time. Accordingly, Brownian motion of chromatin domain can quickly dissociate an aggregate of PF mimicking a TF (cf. Fig. 1.8B with C). An elegant ultrastructural study shows that global downregulation of transcription quickly leads to a strong reduction of the number of PF; a restimulation of cellular RNA synthesis gives rise to a rapid occurrence of PF which parallels that of the newly formed nucleoplasmic RNA within the PR (Nash et al., 1975).

We do not know the true space–time chromatin structure of either a silent or a transcribed gene in vivo, not to speak about the implications of transcription on neighboring chromatin. Some speculations seem justified at
this point, if only to trigger thoughts and new experimental approaches
concerning the still unknown extent of local chromatin dynamics possibly
involved in transcription (as well as in DNA replication and repair).

We will consider two scenarios for the scale and dynamics of chromatin
reorganization and repositioning events possibly involved in transcription.

Although these scenarios are admittedly highly speculative, we wish to
undertake this exercise in order to show how little we still know about
the structural biology and nuclear topography of transcription as one of the
most important nuclear processes.

- **Scenario 1.** *Full decondensation of genes as an essential requirement for transcription.*

  A whole gene present in a higher order chromatin configuration in its
silent state may decondense, prior to transcription, to a looped $\sim 30$ and
$\sim 11$ nm chromatin fiber expanding within the PR or into the interior of
the IC (depending on one’s favored model of nuclear architecture, Fig. 1.2).

This assumption would fit models, which argue for the presence of tran-
scriptionally active genes on extended chromatin loops, but may turn out to
be oversimplified if not invalid to describe the true complexity of gene
regulation. Whereas the ICN model (Fig. 1.2B) seems to argue that a
transcription machinery is built up wherever necessary, we argue that its
built up and spatially fixed predominantly within the PR (Fig. 1.2A). Active
genes may be entirely located within the PR possibly expanding along the
surface of the compact chromatin domain interior, while silent genes are
buried entirely in the interior including its regulatory sequences. Such an
organization may provide a topographical barrier, which helps to prevent a
functionally detrimental or even catastrophic event of transcriptional acti-
vation of permanently silent genes, since transcriptional activation of such
genes would require their repositioning into the PR. In order to work, such
a mechanism would require that such a buried gene is not accessible for the
transcription machinery. Since published data indicate that the interior of
chromatin domains is accessible for individual proteins, it seems plausible
that a factor required for the relocation of a buried gene into the PR can
reach its respective target within the interior of a given domain. Alterna-
tively, we consider the case that a regulatory sequence responsible for the
positional transfer of the buried part of the gene may always be located in
the PR, like the bells at house walls (chromatin domain surfaces) located
along a given street (interchromatin channel), whereas intron and exon
sequences are buried in the domain interior.

- **Scenario 2.** *Transcription involves stepwise gene decondensation and recondensation process.*

  In case that all silent genes independent of the location of the major
chromatin part expose a target site within the PR, this site could become
the starting point for the sequential, stepwise relocation of only a small, fully
decondensed segment of a given gene into the PR during transcription, while the nondecondensed part is still located either directly beneath the PR or even deeper within the compact interior of a chromatin domain. The accessibility of the domain interior for individual proteins involved in the construction of a complete transcription competent machinery is consistent with the possibility that such a machinery can be built up exclusively in the PR since only decondensed chromatin in the PR may show chromatin modifications required for the binding of certain transcription factors essential for its built-up. Scenario 2 argues for a transcription process working hand in hand with a very dynamic de- and recondensation process of a gene’s chromatin in a sequential, stepwise manner. While one piece of DNA is being transcribed, the next segment of the gene is becoming fully decondensed and located into the PR, while the posttranscriptional piece of DNA becomes recondensed and relocated into core chromatin beneath the PR. Accordingly, transcription would involve highly dynamic local chromatin events albeit at scales which cannot be resolved by conventional light microscopy.

For both scenarios a nonrandom organization of the chromatin domain is an essential requirement raising the question of which mechanism may guide the movement of a buried gene or part of it to the domain surface and back into its interior? Finally, it should be emphasized that we do not even know presently whether the PR is the preferential nuclear subcompartment for active genes only or whether it contains both active and silent genes. Observations suggesting the presence of silent PcG genes within the PR provide a case in point (Cmarko et al., 2003).

Whatever scenario may be considered more likely than others, we need to know to what extent certain sequences are nonrandomly exposed at chromatin domain surfaces, while others are located in the interior. We also need to know the circumstances and dynamics with which certain genes or parts thereof may be exposed in the PR and relocated in the interior of chromatin domains. Timescales required for these dynamics may differ strongly between genes which become transcriptionally active on short notice and genes which are kept permanently silent in a given somatic cell type, such as pluripotency genes which become only active during a complex reprogramming event. Since the scale of functionally essential repositioning events may often range below the resolution of conventional light microscopes, attempts to obtain experimental evidence in favor of or against the transcription scenario discussed above can only be based on EM possibly supported by advanced light microscopic approaches breaking the Abbe/Raleigh limit of conventional light microscopy.

Studies of the dynamic nature of nuclear architecture are still in their infancy and many pertinent questions remain to be answered. When are cell type-specific proximity patterns of chromosomes established during development (during mitosis, interphase, postmitotic terminal differentiation)? Can a given proximity pattern be maintained throughout mitosis?
Or is such a pattern lost during mitosis and needs to be newly established during interphase or in postmitotic cells? What kind of mechanism allows different cell types to adopt cell type-specific chromosome proximity patterns? Obviously, direct observations of CT and gene movements in living cells are desirable. Such experiments are possible, but are not easy to perform, since the introduction of visible markers may interfere with normal gene movements. For a comprehensive answer, new, sophisticated approaches which combine 4D (space and time) live-cell studies of nuclei with ultrastructural resolution must be developed.

4.3. Forces responsible for changes of higher order chromatin arrangements

What are the forces which drive short-range (nm scale) and long-range (μm scale) movements of nuclear structures? For short-range movements, Brownian motions may entirely suffice, although even short-range movements may be energy-dependent. The problem of energy dependence is particularly obvious in case of long-range movements of chromatin, which bring together DNA from widely distant parts of a given chromosome or even from different chromosomes. Evidence for the nuclear localization of actin and myosin has put these proteins in the spotlight as potential participants in long-range chromatin movements (Farrants, 2008; Milankov and De Boni, 1993) and their important roles in transcription and possibly in signal transduction were revealed (Hofmann, 2009; Louvet and Percipalle, 2009; Pederson, 2008; Pestic-Dragovich et al., 2000). Nuclear actin/myosin as a nuclear motor machinery has recently been claimed as being involved in the molecular mechanism(s) responsible for large-scale movements of CTs, chromatin domains and genes (Hu et al., 2008; Mehta et al., 2008). Yet, we are still far away from a true understanding of what mechanisms actually drive these movements. In order to achieve an impression of the potential complexity of a mechanism required for “kissing” events in trans, let us consider two scenarios (A and B).

Scenario A: CTs harboring genes, which engage in trans “kissing” events in a given cell type, maintain a strictly nonrandom neighborhood directly favorable for such a “kiss.” Under such conditions, small-scale Brownian motions may suffice to bring sequences into “kissing” contact (here even positional changes as small as 50 or 100 nm may be of great functional significance). When needed, random contacts can be stabilized by protein–DNA interactions. Scenario A does not conform to present data suggesting a strong cell-to-cell variability of CT proximity patterns, but given the relative paucity of pertinent studies it should not be excluded from consideration. In favor of this scenario we need to answer the question as to which mechanisms are responsible to establish cell types with a highly ordered chromatin neighborhood?
Scenario B: Potentially "kissing" sequences are widely separated from each other (typically in the order of several μm). Following mitosis the CT neighborhood arrangements present in daughter nuclei differ largely and unpredictably from the arrangement present in the mother nucleus. Accordingly, two CTs, which were neighbors in the mother nucleus, may be widely separated in its daughters. Since both 3C and 3D FISH experiments provide only snapshots from a population of cell types, transient "kissing" events can be detected only in a minority of nuclei. Long-distance movements of either whole CTs or of giant loops expanding from them (Fig. 1.2D) are necessary to bridge the space between remote CTs. Mechanism(s) necessary to bring about cell type-specific, transient or permanent "kissing" events in trans over long distances must involve major—and as we argue: directed—chromatin movements (Chuang et al., 2006). Recent claims that entire CTs are able to make major movements within periods of half an hour or even less (Hu et al., 2008; Mehta et al., 2008) came unexpected and were so far not supported by direct observations of such rapid movements in living cells.

In case that several "kissing" events between different pairs of homologous or nonhomologous CTs are required, the required mechanism(s) may turn out to be exceedingly complex. To illustrate this point, let us assume that CT neighborhood arrangements are random in a given stem cell population, whereas different somatic cell types attain cell type-specific proximity patterns of chromatin to allow DNA sequence interactions in trans. Trans "kissing" events may be brought about by large-scale movements of entire CTs and/or of giant chromatin loops expanding from the respective CTs. In case that a congression of entire, widely separated CTs is required in order to enable a "kiss" in trans, repositioning would necessarily involve numerous other CTs as well, since CTs, which separate the two potential "kissers," need to move aside. In case of long-range interactions of giant chromatin loops (Fig. 1.2D) (de Laat, 2007; Ling and Hoffman, 2007), physical constraints need to be overcome that will hinder long-distance passages of giant loops through the nuclear space. On their route, giant loops must be able to penetrate through or pass around one or several other CTs forming obstacles between the site of departure and the site of arrival of a gene traveling on a given giant loop. In principle, interchromatin channels could serve as routes for expanding loops to remote nuclear sites (Albiez et al., 2006; Bouchet-Marquis et al., 2006). However, such channels are, of course, not empty but filled with macromolecules and nonchromatin domains, which also provide obstacles for expanding and retracting giant chromatin loops. Models contradicting the existence of the IC, such as the ICN and the GLF model, make the directed passage of giant loops throughout the nuclear space an even more insecure and doubtful journey. In cycling cells, chromosome condensation at the onset of mitosis may create additional mechanical problems for the undisturbed retraction of
such loops toward their home chromosome. We conclude at this point that the topographical implications of transient “kissing” events have not been sufficiently described to date nor has compelling evidence been provided for a direct functional significance of such events.

4.4. Unexplored biophysical properties of nuclei

The biophysical properties of nuclei are another field, which needs to be explored (Kanger et al., 2008; Lavelle and Benecke, 2006; Rowat et al., 2008). Changes of nuclear shape and stiffness during differentiation from stem cells to terminally differentiated cells are well known but not well understood (Pajerowski et al., 2007). Evidence that CTs apparently form 3D higher order chromatin networks (Albiez et al., 2006) may have important, still unexplored implications for nuclear stiffness and mechanotransduction. The latter implies that forces transmitted from the extracellular matrix via the cytoskeleton to the nucleus can alter gene expression in a variety of cell types (Campbell et al., 2008; Gieni and Hendzel, 2008).

The nucleus provides a crowded environment. The role of macromolecular crowding and entropic forces on local chromatin compaction and thus functional states of chromatin is supported by both theoretical considerations and experimental evidence (Hancock, 2004; Hancock, 2007; Richter et al., 2008; Rippe, 2007). This evidence suggests that macromolecular crowding is involved in self-association of polynucleosome chains (Hancock, 2008) and stabilizing CT structure. It seems possible that CTs represent separate phases like those seen in heterogeneous particle mixtures by experiment and simulation. Yet, the interplay of these factors is not well understood with respect to the compaction or decondensation of different chromatin domains containing unique and/or repetitive DNA sequences, nucleosomes with different epigenetic modifications, and different histone variants as well as countless nonhistone proteins. It is essential to combine microscopy with biochemical and biophysical approaches to achieve a better understanding how different nuclear domains are formed, maintained, altered, or degraded in the context of the global functional architecture of the cell nucleus.

4.5. Summary

Although often labeled as “only” descriptive, detailed structural studies are mandatory to provide secure knowledge of evolutionary-conserved main structural motifs and of the extent of cell type- and species-specific features. Such data are indispensable as an essential basis of future research, even if the results cannot be used immediately to generate or test molecular hypotheses on how a given cell type-specific nuclear architecture is generated and maintained. Like extensive comparative DNA sequencing data are
necessary to understand the history of species evolution, comparative evolutionary data of nuclear architecture are an indispensable part of attempts to understand how the present range of epigenomes of cell types within and between species has evolved. The planning of such studies on a sufficiently large scale requires improved bioinformatics tools able to handle the analysis of large amounts of data. In comparison with projects, which focus on the evaluation of molecular mechanisms, chances of getting such “descriptive” projects funded, as well as chances to publish their results in high impact journals, are presently much lower. This situation provides an important drawback for the whole field of cell biology. Without detailed descriptive knowledge on the possible extent of modifications of the nuclear architecture, speculations about mechanisms involved in generating, maintaining, or even reprogramming the myriad of epigenomes present in multicellular organisms are built on insecure ground.

Current experimental approaches to manipulate the nuclear architecture (Finlan et al., 2008; Kumaran and Spector, 2008; Mateos-Langerak et al., 2007) need to be further developed in order to manipulate the location and/or compaction of entire CTs, chromosomal subregions, or single genes with the aim of exploring possible effects of such changes on nuclear functions. These approaches should be pursued together with more sophisticated, quantitative modeling of higher order chromatin and nuclear architecture at large, which takes into account the dynamic interactions of nuclear components including the higher order organization of chromatin and chromatin interactions, the movements of important molecular constituents, such as RNA molecules and proteins with regulatory functions, as well as the assembly and disassembly of functional machineries, nuclear bodies, and splicing speckles (Carrero et al., 2006; SaccoBubulya and Spector, 2002). Only few attempts have been made so far to develop models of individual CTs and their higher order organization, which allow quantitative predictions for experimental tests (Bolzer et al., 2005; Cremer et al., 2000; Kreth et al., 2004b; Munkel and Langowski, 1998; Munkel et al., 1999; Sachs et al., 1995; Shopland et al., 2006), such as quantitative predictions on experimentally induced chromosome aberrations. Such experiments in turn will help to falsify or validate and improve such models (Branco and Pombo, 2006; Friedland et al., 2008; Holley et al., 2002; Kreth et al., 2004a, 2007; Levy et al., 2004).

As important as a valid theoretical foundation for a research strategy are questions concerning the usefulness and limitations of available experimental methods and the development of new methods, which open the gate for new discoveries. In Section 5 we will focus on new microscopic developments and the importance of being earnest about correlative microscopy combining both advanced FM and EM.

Concluding this section, we wish to point out that it is not helpful to emphasize one approach on the cost of another. Understanding the
functional nuclear architecture is a huge task for many years to come. While many tools have still to be developed, integrative approaches are the key to success. It is obvious that approaches dominated by a single method lead to insufficient answers or fail entirely (Robinson et al., 2007). The importance of multidisciplinary approaches can be underlined by progress in understanding the structure–functional relationships of nucleoli, probably still the most studied nuclear compartment/entity to date (McKeown and Shaw, 2009), the nucleosome (Corpet and Almouzni, 2009; Luger et al., 1997), and the NPC (Alber et al., 2007b; Beck et al., 2007; Dange et al., 2008; Maco et al., 2006). This progress has become possible by the integration of structural analyses with a range of complementary methods as well as data translation into models for spatial restraints, optimization, and ensemble analysis (Alber et al., 2007a). Despite this progress there are still many open questions and conflicting ideas how these so far best studied nuclear structures really function.

Following these examples, functional and structural data from cells and their nuclei must be obtained by all possible means from the level of single molecules to a multitude of higher order chromatin structures to nonchromatin domains, such as nuclear bodies and interchromatin granules (splicing speckles) and last but not least to the macromolecular machineries involved transcription, RNA-processing, replication, and repair. The main strength of a proper combination of state-of-the-art microscopic methods lies in its potential to elucidate the dynamic topography of all nuclear constituents at the single cell level, but this can only be exploited when imaging methods are part of a proper context of other methods.

5. Quantitative Microscopic Analysis of Nuclear Architecture

This section was written in order to provide basic information about special advantages and limitations of present approaches and to point out some new developments. While this information is, of course, limited, we hope that it provides an idea of the complexities of advanced cell imaging techniques and their potential for future studies of the functional nuclear architecture.

5.1. Electron microscopy

Compared with light microscopy, the most obvious advantage of EM is its much higher resolution, which is in the range of 0.5 nm in the case of TEM and a few nanometers in the case of scanning EM, while the most obvious disadvantage is the restriction to fixed cells only. Methods for the
visualization of DNA or RNA synthetic sites use either tritium labeling and autoradiography or halogenated precursors and immuno–EM. In both cases the specimen is represented by ultrathin sections of a chemically fixed or cryofixed cellular sample either subsequently embedded into plastic resin or cryosectioned. In the former, visualized label reflects the intracellular distribution of nucleic acids localized by virtue of a radiation-sensitive photographic emulsion superposing the specimen and photographically treated to obtain the final signal. Although offering a lower detection resolution than immuno–EM, it is especially convenient for subsequent kinetic analyses of distribution of different components in the cell.

As to the immunocytochemical detection, it is important to be aware of the difference of structural preservation between pre- and postembedding labeling approaches:

1. In a preembedding protocol a given cell is embedded in resin for subsequent ultramicrotomy only after completion of immunocytochemical reactions with antibodies against specific epitopes and, for example, a secondary antibody coupled with a convenient marker, typically giving rise to a product of an enzyme–substrate reaction. Accordingly, ultrathin sections studied by EM reveal specific signal (and unwanted background) throughout the whole section. The major disadvantage of preembedding protocols stems from the fact that procedures needed for performing immunocytochemical labeling before embedding can damage the ultrastructure of the cell to an extent which is difficult to control precisely. Permeabilization steps with detergents are often required to obtain probe penetration into the sample, which may give rise to extraction or displacement of cellular components. Structural damage due to DNA dispersal is particularly obvious in the case of in situ DNA hybridization procedures, which require a DNA denaturation step (Solovei et al., 2002). In all cases where it is necessary to generate cytochemical signal from the entire depth of each section, the preembedding approach cannot be avoided, but it is necessary to emphasize here the danger, already discussed above, of structural artifacts generated by preembedding in contrast to postembedding protocols.

2. In a postembedding protocol the fixed cell is first embedded in resin, and ultrathin sections are prepared before an immunocytochemical method is applied. In addition, chemically fixed and cryosectioned material may also represent a suitable alternative (Fakan et al., 1984). The major advantage of this approach is due to the unparalleled preservation of fixed specimens. Epitopes for binding of specific antibodies or nucleic acid sequences for in situ hybridization with specific DNA or RNA probes are detected at section surfaces exposed by ultramicrotomy. Structures embedded within the section are not affected by labeling and detection procedures and thus remain well preserved. Compared with
preembedding protocols, this generally gives rise to lower signals. However, preembedding labeling is achieved at the cost of a much lesser and sometimes unacceptable quality of ultrastructural preservation and, consequently, of localization precision. Moreover, reactive sites located inside dense compact cellular compartments may not be accessible for probes, while they are uncovered by sectioning and consequently accessible when the reaction takes place directly on ultrathin sections. For the analysis of finest details of structures revealed by means of ultrastructural cytochemistry, the postembedding approach is recommended.

Ultrastructural cytochemical studies of the cell nucleus (Biggiogera and Fakan, 2008) usually require chemical fixation with aldehydes followed either by dehydration and embedding into different kinds of resin, or by cryoultramicrotomy making use of a cryoprotectant (Tokuyasu, 1973). Protocols using cryofixation, cryosubstitution, and resin embedding excluding any conventional fixative can also routinely be applied (von Schack and Fakan, 1993), allowing one to work under optimal conditions with regards to the detection of various molecular components of the cell and its activities. In this context, it is interesting to mention that all these different methods, including cryoelectron microscopy of vitrified sections (Bouchet-Marquis et al., 2006), have yielded similar basic morphological features regarding the compartmentalization of the cell nucleus.

5.2. Far-field fluorescence microscopy with conventional resolution

Compared with EM the most obvious advantage of FM is the possibility of live-cell observations, while its much lower resolution represents the most obvious disadvantage. Conventional E(2) (Epi) FM and CLSM (Confocal Laser Scanning fluorescence Microscopy) are limited to an optical resolution of about 200 nm laterally and 600 nm axially. Vital fluorescence markers or tags have offered the possibility to follow individual labeled cellular components and to analyze their interactions in intracellular molecular pathways in living cells down to the level of single molecules (Grunwald et al., 2008). The caveat and limitation of these studies is the requirement that fluorescence tags must not alter the cellular functions under scrutiny. Structural deterioration must be taken into account in DNA in situ hybridization experiments. Heat denaturation of DNA is typically used as a means to render target DNA strands accessible to hybridization with single-stranded DNA probes. TEM of cells after heat denaturation revealed pronounced, although locally restricted dispersal of DNA. As a consequence, 3D FISH combined with new fluorescent microscopic approaches breaking the Abbe limit (see below) or with subsequent TEM analysis (Solovei et al., 2002) is a priori problematic as an approach to study the
ultrastructure of chromatin. On the other hand, the relative 3D positions of CTs and ~1 Mb chromatin domains recorded in living cells after pulse-labeling with fluorochrome-conjugated nucleotides during S-phase hardly changed after 3D fixation procedures with buffered formaldehyde (Solovei et al., 2002). When these fixed cells were subjected to a chromosome paint experiment, changes of the relative positions of foci were noted in the order of a few hundred nanometers. Accordingly, we argue that 3D FISH is a useful approach to detect differences of the architecture of individual CTs, as well as differences of CT arrangements down to the limits of conventional light microscopic resolution.

Confocal laser scanning fluorescence microscopy (CLSM) has provided a tool for the routine recording of stacks of light optical serial sections, which are perfectly suitable for 3D reconstructions. The application of deconvolution algorithms seems mandatory for light optical sections obtained by EFM, but can also improve the quality of CLSM sections considerably. The rational choice, which of various deconvolution procedures should be preferred for a given case, is often hampered by a typical lack of knowledge about what the true features of imaged structures may be. For example, an observer may prefer structures with clear borders distinguishing these structures from their neighborhood. The choice of a deconvolution protocol, which enhances such borders, however, may be misleading. The more already is known by other studies about the true features of a structure, the easier it will be to make a reasonable choice.

Multicolor 3D FISH has allowed to visualize multiple targets with different colors, such as the CTs from all pairs of homologous autosomes and the two sex chromosomes X and Y in human diploid fibroblast nuclei (Bolzer et al., 2005). A major disadvantage of CLSM is its limited recording speed, which does not provide the temporal resolution necessary to monitor very rapid biological processes in living cells, for example, the dynamics of microtubule assembly and disassembly. This disadvantage has been overcome by the development of spinning disk confocal microscopy. These instruments contain a thin wafer with hundreds of pinholes arranged in a spiral pattern, called a spinning disk and in contrast to a conventional CLSM where an image is recorded point by point, spinning disk confocal microscope allows the simultaneous recording of fluorescence emitted by many object points. This allows a much higher speed for the recording of a very thin optical section. AOTFs (acoustooptical tunable filters) offer a micro-seconds switch of excitation wavelengths.

It is often important to decide whether two fluorescent domains are fully separated from each other, whether they just touch each other or whether they—within the resolution limits of the microscopic equipment—partially or even fully colocalize with each other. We start with the apparently most trivial request to obtain a random sample for statistical analysis. In case of 3D FISH experiments, some 20–50 cells are typically chosen from the whole
cell population or from a given cell type present in this population (or in case of more specific demands from cell types at a given stage of interphase or postmitotic differentiation). As a major danger, one should be aware of an unnoticed biased selection of cells in quantitative 3D FISH assays, which may be an important source of misleading conclusions. Only 3D FISH preparations with a very high yield of analyzable cells (>95%) should be evaluated. Criteria for rejection of cells from further evaluation must be carefully outlined in advance and the reasons for rejection during evaluation should be presented. For obvious reasons, one should strictly avoid looking on FISH signal patterns when deciding which cells are chosen for detailed evaluation. This can be achieved by looking first on the DNA counterstain and rejecting cells with apparently damaged nuclei. Only thereafter filter sets should be changed to record the FISH signals. The use of a random number generator to choose coordinates for evaluation on a given slide, likely provides the best possible assurance against sampling errors, which may lead to wrong statistical conclusions.

Secondly, one should keep in mind the limitations of the resolution of the microscopic equipment used for analysis. A conventional confocal microscope, for example, has an optical resolution of about 200 nm in x/y- and 600 nm in z-direction (Stelzer, 1995). Small domains apparently overlapping in a light optical section may be separated by a corridor of another nuclear compartment when viewed with the much higher resolution of the electron microscope. According to the Abbe limit of light optical resolution, two fluorochromes closer to each other than the resolution limit of about half the wavelength used for imaging (Abbe 1873; Rayleigh 1903) cannot be discriminated as separate objects as long as they emit light of the same spectrum. An important implicit assumption of this statement is that the two objects emit light simultaneously. We will see below that this limitation can be overcome in case that the two objects emit light with different spectral signatures, e.g. in a sequential manner (Spectral Precision Distance Microscopy, SPDM; see below). The choice of fluorochromes, which emit fluorescent light of different wavelengths, provides another possibility to overcome this limitation. Here, the emission of the two fluorochromes can be recorded separately by use of an appropriate filter, and the 3D position of the intensity gravity center of the fluorescence can be recorded separately for each fluorochrome with a precision in the nanometer range. This allows high precision 3D distance measurements between the two intensity gravity centers with a resolution clearly far below the Abbe limit (Bornfleth et al., 1998; Cremer et al., 1999). As a caveat of such measurements, one has to take into account that chromatic aberrations of the microscope objective, which may be quite different for different wavelengths, lead to chromatic shifts, which need to be very carefully corrected, but this can be done with high precision (Esa et al., 2000; Rauch et al., 2008). When all the mistakes lurking around quantitative 3D light
microscopy are carefully avoided, conventional FM in the SPDM mode allows high precision 3D distance measurements as small as 50 nm. In contrast to this improvement of 3D distance measurements between a small number of closely adjacent objects, quantitative measurements of the true shape and volume of objects far below the Abbe limit of resolution is still impossible with conventional FM, including CLSM and one cannot decide to which extent the periphery of two objects may overlap. It is even difficult to perform precise measurements of volumes of fluorescent objects with dimensions clearly above the Abbe limit, such as painted CTs, since these measurements are threshold-dependent. When using paint probes for different CTs, it is important to be sure that they are of comparable quality in covering their target CT. Furthermore, the use of an excess of unlabeled Cot1-DNA to prevent the unwanted hybridization of labeled repetitive probe sequences, means that only a fraction of the entire CT in question can be targeted even with the best chromosome paint probes.

The determination of an appropriate threshold able to distinguish specific fluorescence that belongs to a given object, for example, a splicing speckle, from background fluorescence is a difficult problem. Attempts to solve this problem are typically based on segmentation procedures using a threshold, which apparently distinguishes between pixels/voxels with signals above the threshold arguably belonging to the fluorescently stained object, while pixels/voxels below the threshold are attributed to background. Depending on the choice of the threshold with its subjective component, the uncritical interpretation of the results can lead to erroneous conclusions. The visualization of speckles with antibodies against SC-35, a protein clearly present in speckles, provides a case in point. Using EFM, Fay et al. (1997) investigated the nuclear distribution of the signal generated by indirect immunofluorescent detection of an antibody against a phosphoepitope of the related splicing factors, SC-35 and SF2/ASF (Fay et al., 1997). As expected they observed a speckled pattern, yet also detected that 70–80% of their signal was found as a diffuse nuclear signal. Most of the latter, however, was not just background but apparently represented the SC-35 location in PFs in the PR (Spector et al., 1991), where cotranscriptional splicing occurs. Simple removal of this “background” would have supported the erroneous conclusion that SC-35 is a factor specifically contained in speckles but not elsewhere. Still, one may wish to count the number of speckles which remain as distinct clusters after eliminating “background” signal. If one does so, one should keep in mind that a fraction of the smaller speckles may not correspond to the interchromatin granule clusters defined by EM studies but rather represents aggregates of closely adjacent PFs. This example should just set a warning light that the choice of a threshold, which is not based on objective observer-independent criteria but rather on the experience, not to say the prejudice, of an investigator can lead to wrong interpretations. As a primer for beginners: the conclusion that colocalization
of two proteins is proven when indirect fluorescence antibody staining of the two proteins in green and red color results in an image with yellow overlap is not considered state-of-the-art, although such evidence is widely published. The choice of a threshold procedure applied for removal of background must be carefully justified and documented. A threshold may be set for an entire observation field or various thresholds may be used for different parts of the field. The latter approach needs a clear justification. This, for example, is the case when background fluorescence is very inhomogeneously distributed and much higher in some part of the observation field than in another. For a valid interpretation it is important to consider the choice of an appropriate threshold and other parameters of the imaging procedures in the light of as much information as possible about the biochemical nature of visualized nuclear domains, especially when they are considered as novel. The validity of the interpretation stands or fails with the specificity of the employed antibodies used and may be complicated by the dynamic participation of a given target proteins in different structures. Beyond the microscopic information per se, all this knowledge is an indispensable part of the interpretation of imaging data. Needless to say that background is also an important problem in EM studies. When performing a quantitative EM analysis, for example, the quantitative evaluation of immunogold labeling on ultrathin sections, one has to analyze the labeling density on resin outside the cells or tissue material as a reference for background signal.

5.3. Far-field fluorescence microscopy with resolution beyond the Abbe limit

The optical resolution of a conventional far-field light microscope is given by the “Abbe-Rayleigh limit” (Abbe, 1873; Rayleigh, 1903), corresponding to roughly half the wavelength in the lateral direction and one wavelength in the axial direction. Recently, new laseroptical superresolution instrumentation has made it possible to extend the spatial analysis of cells far beyond the resolution limit of conventional FM (Table 1.1). This breakthrough does not invalidate the Abbe-Rayleigh theory of light microscopic resolution, which still describes an unsurpassable limit for conventional light microscopes. It shows, however, the limitation of this theory, which does not hold for certain laseroptical approaches which make use of possibilities beyond the technical limitations and imagination of Abbe’s time. This breakthrough has opened unprecedented opportunities for cell imaging (Cremer et al., 2006, 2009; Hell, 2009). The limit of light optical resolution is no longer given by the wavelength of the light used to generate an image, but by photon statistics: in order to resolve a structure with dimensions much below the wavelength of the fluorescent light emitted by a given structure with nanoscale dimensions (microscopy), it is necessary to record a sufficient number of
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- Hell (2007, 2009)
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- Gunkel et al. (2009)
- Baddeley et al. (2007, 2009a), Failla et al. (2002a,b, 2003), Spoerri et al. (2004)
- Heilemann et al. (2008), Rust et al. (2008), Steinhauer et al. (2008)
- Cremer and Cremer (1978), Hell et al. (1994), Hanninen et al. (1995)
photons from the object. The precision with which the position and hence the distance between individually resolved neighboring objects can be measured is thought to be a better criterion to express optical resolution (Albrecht et al., 2001; Van Aert et al., 2006). It is clear that this problem is particularly severe in the case of live-cell imaging. Depending on the dynamics of a recorded structure with respect to changes in shape and position, it may become impossible to record sufficient photons at individual time points. Understandably, the new approaches with Abbe limit breaking resolution described below, typically require fixed cells, although it has become possible to develop approaches which overcome the Abbe/Rayleigh resolution limit for live-cell imaging as well.

5.3.1. 4Pi confocal laser scanning microscopy

Using two opposing high numerical aperture (NA) lenses to concentrate two opposing laser beams constructively in a joint focus, confocal laser scanning 4Pi-microscopy (Baddeley et al., 2006; Egner et al., 2002; Hanninen et al., 1995; Hell et al., 1994) has become an established “optical nanoscopy” method, allowing an axial optical resolution down to the 100 nm regime, that is, about 6–7 times better than in conventional CLSM. The principal idea behind 4Pi-microscopy is to narrow the diameter of the focused laser beam at least in one dimension below the values possible if the beam is focused from one side only (as it is the case in a conventional confocal microscope). Such a procedure might be called “Point Spread Function Engineering.” Conventional lenses can possibly be replaced in the future by other means to focus light to a very small spot size. In the 1970s, C. Cremer and T. Cremer proposed a laser scanning microscope with improved optical resolution based on the use of a “4” (4Pi) point hologram” as focusing element. It was assumed that such a hologram might be generated using a point-like source with a diameter much below the wavelength of the light emitted from such a source or produced according to numerical calculations (Cremer and Cremer, 1972, 1978). This hologram should become the decisive optical element of a “4” (4Pi) laser scanning microscope to focus laser light from all sides (“4Pi—geometry”). A structure with dimensions above the point-like illumination spot could then be analyzed by “point-by-point” imaging of the fluorescence excited in this structure by the illumination spot. The possible optical resolution of this approach would depend on the smallest focal dimensions, which can be realized by the 4Pi point hologram. An ideal 4Pi point hologram might be approximated by a number of plane point holograms. Even in case that the spherical angle realized would be still considerably smaller than 360°, this new type of a scanning microscope may generate a smaller focus in all three dimensions as compared to microscopes equipped with one or two opposite conventional objectives.
Considering the theoretical limitations of holographic focusing, one has to take into account that in the immediate vicinity of a light source much smaller than the wavelength of the light radiated by this source, effects such as the polarization properties of the electromagnetic field start to play a dominant role. In the case that a hypothetical spherical wavefront of constant intensity is focused from all sides (“4Pi geometry”) in a medium of constant refraction index, theoretically a spot diameter of about 1/3 of the wavelength may be obtained (Hell, 2007). Hence, the optical 3D resolution would be improved by a similar factor. For an excitation wavelength of 488 nm and a refraction index of 1.4, a spot diameter of about 1/3 of the wavelength means a limiting isotropic spot size around 100 nm. In contrast to the observation volume achieved by a conventional CLSM (200 × 200 × 600 nm³), this would mean a 24 times smaller observation volume (100 × 100 × 100 nm³) and a correspondingly improved 3D resolution.

Focusing by a spherical wavefront of constant intensity and linear polarization is just one of the many possible illumination conditions. Arguably, this estimate may not provide the theoretical limit of holographic focusing. For example, using a radially polarized laser beam, the experimentally observed spot size for an NA = 0.9 was observed to be about 35% below the theoretical limit for linearly polarized light; using specific photosensitive layers, even smaller spot sizes can be reached (Dorn et al., 2003). Similar effects should be obtained if the objective lens used in these experiments for focusing would be replaced by an appropriate plane point hologram. Instead of a single point hologram, in the 4Pi case 3D assemblies of plane holograms may be considered, where each single hologram is designed to contribute to the formation of a focus much below the dimensions, which can be achieved with conventional optics. Pulsed laser light with different wavelengths, including novel possibilities of attosecond laser physics (Silberberg, 2001) might be used to generate the appropriate spatial, temporal, and phase distribution of the illuminating field necessary to generate the desired focal point or pattern.

Holograms might be designed, for example, to replace conventional optics with long working distances in laser scanning microscopes. The distance of the focus generated by a given hologram arrangement can be much larger than possible with any conventional system of lenses without compromising the desired characteristics of the focus. This advantage makes it possible to develop assemblies of holograms as new focusing devices from all directions of the space with geometries and hence spatial light distributions which are impossible to realize with conventional high NA objective lenses. The present state of 4Pi-microscopy provides a point in case. While it is possible to align two high NA objective lenses opposite to each other in order to generate the interference pattern necessary to obtain an increased optical resolution, the geometry of these objectives precludes the simultaneous use of additional high-aperture objectives at many different angles. Such a limit does—at least in principle—not exist for a 4Pi scanning
microscope based on an assembly of holograms. Considering the possibilities and technical limitations to develop 4Pi holographic focusing devices instead of conventional optics, we feel presently not able to come to a safe conclusion regarding the technical limitations of “4Pi-focusing” with the purpose of generating a scanning-based fluorescent image with improved optical resolution. In principle, the smaller the exciting spot diameter, the better the effective optical resolution. We believe that the possibilities of 4Pi holographic focusing devices have not been fully explored and deserve further theoretical and experimental studies. Below, we consider stimulated emission depletion (STED) microscopy as a most elegant and successful approach to experimentally achieve a focal region with dimensions much below the Abbe limit. It might become possible to establish 3D STED microscopes equipped with holograms instead of conventional optics.

5.3.2. Stimulated emission depletion microscopy
STED microscopy was first conceived and realized in the 1990s (Hell and Wichmann, 1994; Schrader et al., 1995). The basic idea of STED microscopy is to reduce the size of the region of a biological object, such as a cell, in which fluorescence is excited by a very short excitation pulse. This goal is achieved by a “depletion” pulse (“STED pulse”), which follows immediately after the excitation pulse and acts in the vicinity of the center of the fluorescent region. This is done in such a way that the STED pulse forms a ring around the center of the fluorescent region. As a consequence, fluorescence is only detected from a much smaller region. Due to the scanning mechanism, the position of this smaller fluorescent region can be identified with an accuracy down to the 1 nm range. Accordingly, the recorded fluorescence signal can be assigned to this smaller region further improving the optical resolution. STED microscopy was the first successful implementation of more general concepts of focused beam superresolution approaches like RESOLFT (REversible Saturable Optical Fluorescence Transitions) or “ground state depletion microscopy” and has already presently found numerous applications in high resolution cell biology (Hell, 2007).

In principle, there is no limitation with respect to the lateral resolution, which can be obtained by STED microscopy except for the requirement that an amount of photons must be recorded from any given illumination point sufficient to generate an image. Using a combination with 4Pi-microscopy, STED has recently been realized also in three dimensions with a 3D resolution in the lower nanometer range (Schmidt et al., 2008).

5.3.3. Structured illumination microscopy (SIM)
Improvements of light-optical resolution beyond the Abbe/Rayleigh limit achieved in the case of 4Pi confocal microscopy and STED microscopy became possible by the application of “just physics.” This means that in principle a better resolved image is obtained without a need for further complex data processing. Other modes of circumventing the Abbe/Raleigh...
limit have been implemented by a combination of advanced optical and computational methods. One of these additional possibilities is to illuminate the object with an appropriate pattern of light; moving either the object or the pattern, at each relative position an image of the object is taken by a highly sensitive CCD camera. Using complex but well-established algorithms in the Fourier space, from such images it becomes possible to reconstruct an image of the object at enhanced effective optical resolution (Frohn et al., 2000, 2001; Gustafsson, 2005; Gustafsson et al., 1995, 1996; Heintzmann and Cremer, 1999; Schermelleh et al., 2008). Recently, even a 3D optical resolution enhancement and its application to the analysis of structures at the nuclear envelope has become possible (Fig. 1.9).

5.3.4. Spatially modulated illumination microscopy (SMIM)
SMIM (Baddeley et al., 2007; Failla et al., 2002a,b, 2003; Spoeri et al., 2004) is another possibility to use structured illumination to improve spatial analysis. It is based on the creation of a standing wave field of laser light (Bailey et al., 1993). This can be realized in various ways, for example, by focusing coherent light into the back focal planes of two opposing objective lenses of high NA. The fluorescence-labeled object is placed between the two lenses and moved axially in small steps (e.g., 20 or 40 nm) through the standing wave field. At each step fluorescence is registered by a highly sensitive CCD camera (Fig. 1.10). This procedure allowed to measure the diameter of individual fluorescent objects down with sizes of about 1/15 of the exciting wavelength, that is, down to a few tens of nanometers for visible light, and to determine axial distances between “point-like” fluorescent objects at the lower nanometer scale with a precision in the 1 nm range (Albrecht et al., 2002). Several biophysical application examples indicated the usefulness of SMI-''nanoscopy’’ for the study of the size of individual chromatin regions (Hildenbrand et al., 2005; Mathee et al., 2006; Reymann et al., 2008) and of TFs (Martin et al., 2004). In the latter case, the SMI results obtained were comparable with EM images obtained from the same type of specimens, revealing mean size differences in the 30 nm range. In other SMI-nanoscopy experiments, high throughput precision size measurements of replication foci were performed (Baddeley et al., 2009a).

5.3.5. Spectral precision distance/position determination microscopy (SPDM)
SPDM and related techniques (Bornfleth et al., 1998; Cremer et al., 1996, 1999, 2002; Heilemann et al., 2002, 2004; Lacoste et al., 2000; Schmid et al., 2000; van Oijen et al., 1999) are far-field FM approaches based on labeling of neighboring “point-like” objects with different spectral signatures, spectrally selective registration, and high precision position monitoring, that is, a method of “spectrally assigned localization microscopy” (SALM). Combined with careful calibration of optical aberrations, this allows the measurement of positions and mutual distances between
“point-like” fluorescent objects in a range far below the “Abbe limit” of distance resolution for objects of the same spectral signature (~ 200 nm).

“Proof-of-principle” examples for the application of SPDM in nuclear genome structure research have been the analysis of the BCR-ABL region correlated with chronic myeloid leukemia (Esa et al., 2000); of conformational differences in the 3D-nanostructure of the immunoglobulin heavy-chain locus, a hotspot of chromosomal translocations in B lymphocytes (Esa et al., 2001); of the nanostructure of imprinted gene domains in human interphase nuclei (Rauch et al., 2000, 2008); or of the distribution of genes in the active and inactive X-CT (Dietzel et al., 1999).

Figure 1.9 Structured illumination microscopy (SIM) in three dimensions. Top: Scheme of 3D-SIM: by using a diffraction grating, an illuminating sine wave pattern is produced in the object space. At each axial (z) position, five different phases of the sine wave pattern are recorded. Three image stacks are registered with the diffraction grating sequentially recorded into three positions 60° apart. This allows to calculate a 3D-SIM image with enhanced details. Bottom: Comparison of a deconvolved light optical nuclear midsection obtained with a conventional laser confocal scanning microscope (left) and a light optical midsection obtained with 3D-SIM (right). Simultaneous imaging of DNA, nuclear lamina, and nuclear pore complex (NPC) epitopes was performed in C2C12 cells following labeling with antibodies against lamin B (green) and NPC epitopes (red). DNA (blue) was counterstained with DAPI. Enlargements of the periphery of the whole 3D SIM nuclear image suggest channels starting at nuclear pores and permeating through the lamina and between chromatin clusters (cf. Fig. 1.4). These details are not recognizable in the confocal midsection. From Schermelleh et al. (2008), reprinted with permission from AAAS. (See Color Insert.)
In these early SPDM applications, differences in the fluorescence emission spectra were used as spectral signatures. Generally, SPDM (SALM) requires that in a given observation volume (defined, e.g., by the full-width-at-half-maxima (FWHM) of the Point Spread Function of the microscope system used), there is just one object with a given spectral signature to be measured at a given time, a condition referred to as “optical isolation.” Since the reasonable number of useful different emission spectrum-based signatures is still limited (presently \( \sim 7 \)), this means that clusters of numerous fluorescent...
molecules of the same type (e.g., 400 molecules in an observation volume of $0.2 \times 0.2 \times 0.6 \, \mu m^3$) cannot be resolved in this way. The use of differences in the emission spectra, however, is only one of the many ways to realize the “photon sorting” required for the localization of just one molecule in the observation volume at a given time: already in the original SPDM concept, spectral signatures were conceived to include also other “photon sorting” modes like fluorescence lifetimes, photoluminescence, and stochastic-labeling schemes to allow photophysical discrimination (Cremer et al., 1996, 2002).

To realize such “monocolor” spectral signatures, “proof-of-principle” experiments have been performed on the basis of fluorescence lifetimes to measure localizations of single molecules and the distance between them in a range down to few tens of nm (Heilemann et al., 2002, 2004). In this way, SPDM measurements allowed distance determinations too large for FRET techniques but considerably below the optical resolution of conventional, confocal, or 4Pi-microscopy.

Since then, a number of conceptually related SALM methods have been described, such as BLINKING, FPALM, PALM, PALMIRA, SMACM, STORM, dSTORM etc. (Betzig et al., 2006; Biteen et al., 2008; Geisler et al., 2007; Heilemann et al., 2008; Hess et al., 2006, 2007; Huang et al., 2008; Juette et al., 2008; Lidke et al., 2005; Rust et al., 2006).

Single molecule microscopy has become possible by CCD cameras with sensitivity sufficient for the routine imaging of single fluorochromes. This method has already been applied to trace structures labeled with a single fluorochrome in living cells (Grunwald et al., 2008; Lange et al., 2008; Seisenberger et al., 2001). Although in various SALM methods, the lateral ($x,y$) localization of single molecules (object plane perpendicular to the optical axis) has been firmly established, the problem of localization along the optical axis ($z$) has proven to be challenging. To obtain 3D reconstructions of the labeled objects (i.e., the $x,y,z$ coordinates of the fluorescently labeled molecules), various approaches have been applied. One solution is to use confocal laser scanning or confocal laser scanning 4Pi-microscopy (Esa et al., 2000; Rauch et al., 2000, 2008; Schmid et al., 2000; Hueve et al. 2008) to obtain the 3D positions of the objects. Another possibility is based on the 3D information within the laterally acquired signal. Since all light-emitting molecules are “point-like” compared with the wavelength used, one can assume that they all are imaged in the same way. The fact that out-of-focus objects appear more blurred and using a PSF which is not symmetric along the optical axis can be used to localize photon-emitting sources in all spatial dimensions. If the propagation path of the electromagnetic waves is well known, under ideal registration conditions the accuracy of the axial SMI localization ($z$) is restricted only by the number of photons detected (Albrecht et al., 2001), analogous to other microscopy types of localization (Cremer et al., 1999).

Using common photoactivatable or photoswitchable fluorophores in combination with biplane detection (Juette et al., 2008) or a systematically modified
detection PSF (Huang et al., 2008), a 3D single molecule localization accuracy of about 60–80 nm FWHM was recently achieved.

Recently, it was demonstrated that using SPDM with physically modifiable fluorochromes (SPDM$_{phymod}$) conventional fluorochromes such as Alexa 488 or Alexa 568 and 647 (Baddeley et al., 2009a,b; Lemmer et al., 2009b,c; Reymann et al., 2008), the green fluorescent protein variant YFP (Lemmer et al., 2008), or other standard variants (GFP, mRFP) can be used for nanoimaging of cellular nanostructures (Fig. 1.11) (Gunkel et al., 2009). Here, dual color localization microscopy (2CLM) is used under conditions, which result in “reversible photobleaching” of the respective fluorophores (Kaufmann et al., 2009; Lemmer et al., 2008; Reymann et al., 2008). In principle, this means switching of the molecules from a “bright” fluorescent state into a metastable “dark” state (Patterson and Lippincott-Schwarz, 2002; Sinnecker et al., 2005). From this state they return to the “bright” state by a stochastic process, which can be described by fluorescent lifetimes of the ensemble in the order of several seconds. Simple considerations in

![Figure 1.11](Downloaded Image) Comparison of conventional epifluorescence microscopy and nanoimaging of mRFP-tagged histones H2A (red) and GFP-tagged Snf2H transcription factors (green) within a human U2OS nucleus (Gunkel et al., 2009). (A) Conventional epifluorescence image. (B) 2CLM image, here the position of individual mRFP-tagged H2A histones and GFP-tagged Snf2H transcription factors is visualized as a single dot with a size representing the individual localization accuracy. (C)–(E) display Enlargements from the boxed regions in (A) and (B), respectively. Note that the magnification of the conventional image is empty, that is, it does not provide better resolved structural details in contrast to (D) and (E). Scale bars are 2 μm in (A) and (B), 500 nm in (C) and (D), and 100 nm in (E). (See Color Insert.)
analogy to radioactive decay show that such large lifetimes allow highly resolved localization microscopy imaging (Cremer et al., 2010). In contrast to SALM methods such as FPALM, PALM, or STORM based on two frequency photoswitching (Betzig et al., 2006; Hess et al., 2006; Huang et al., 2008), only one laser line per fluorochrome type is required to induce fluorescence/luminescence lifetimes in a given fluorochrome on the second timescale. The burst (or “flash”) of photons, which is emitted from a single fluorophore, allows to determine its position with nanometer accuracy under the condition of optical isolation, that is, the conditions must be such that all other similar fluorophores, which are located closer to the emitting fluorophore than the Abbe limit, should not emit photons of the same spectrum at the same time. We recently showed that reversible photobleaching can be achieved by using an excitation intensity in the 10 kW/cm² to 1 MW/cm² range (Baddeley et al., 2009). For example, Fig. 1.11 shows chromatin visualized by mRFP-tagged H2A together with the location of single GFP-tagged ATPase subunit Snf2H molecules (Gunkel et al., 2009), which define a certain class of chromatin remodeling complexes (Becker and Horz, 2002; Cairns, 2007; Rippe et al., 2007). The results indicate the feasibility to reveal details on the interaction of remodeling complexes with chromatin at unprecedented effective resolution.

SPDM in combination with SMI along the optical axis is a further method to achieve 3D localizations of individual molecules and a corresponding 3D effective optical resolution. Using appropriately labeled cellular structures, a lateral effective optical resolution of 10–20 nm was realized together with an axial effective optical resolution around 30–50 nm. Thus, an overall 3D effective optical resolution around 40–50 nm was achieved, corresponding to about 1/10 of the wavelength used (Lemmer et al., 2008).

In summary, novel developments in laser-optical nanoscopy will reduce the gap in resolution between ultrastructural methods (Angstroms to a few nanometer resolution) and visible light far-field microscopy (conventionally hundreds of nanometer resolution).

5.3.6. Perspectives for in vivo imaging at the nanometer scale
Laser optical nanoscopy has typically been performed on fixed cells in order to demonstrate the achievable optical resolution. Although the application of these techniques to live-cells imaging is still in its beginning, some preliminary results indicate its feasibility. For example, Westphal et al. (2008) analyzed the movement of fluorescently labeled synaptic vesicles in living cells recorded with STED microscopy at a rate of 28 frames/s and an optical resolution around 60 nm laterally. In this study the cross-section area of the focal STED spot was reduced about 18-fold below the conventional diffraction limit of about 260 nm. In another application (Nagerl et al., 2008), STED microscopy was used for live-cell imaging of dendritic spines to dissect synaptic vesicle movement at video-rates.
Due to the scanning mechanism, high-speed nanoimaging in STED requires small regions of interest (in the few micrometer range). In contrast, the various modifications of SALM have the potential for in vivo nanoimaging of large cellular areas (up to $100 \times 100 \mu m^2$; in combination with new optical techniques, even larger ones). For example, Hess et al. (2007) used FPALM to study the dynamics of hemagglutinin cluster distribution in membranes of living cells at 40 nm effective optical resolution to discriminate between raft theories. Preliminary experiments have demonstrated that live-cell imaging is also possible using SPDM with a physically modified fluorophores (SPDMphymod) approaches: In live human cells, the distribution of fluorescent-labeled membrane proteins was registered at an effective optical resolution of few tens of nanometers (Y. Weiland, P. Lemmer, C. Cremer, unpublished observations). In the SPDMphymod experiments, a CCD camera was used allowing to register 15–20 frames/s. The use of high-speed, back-illuminated CCD cameras allowing to register up to 1000 frames/s will highly extend the possibilities of this approach in live-cell imaging applications.

5.4. Importance of correlation microscopy for new ways to realize an old concept

As described above, microscopic analyses of living or fixed cells have their own advantages and limitations, including the possibility of artifacts, which may result from necessary pretreatments of cells prior to microscopy and from limitations of the chosen microscopic method itself. The important point is that available FM and EM approaches are complementary in different ways. Therefore, for a comprehensive, sequential investigation of the same cell, these techniques can and should be combined to add up the advantages and to compensate for the disadvantages of the exclusive use of each method. Correlative microscopy based on the possibility to identify the same structure and its position first with one type of microscopic approach and then with another has opened the way for a wide spectrum of novel applications (Albiez et al., 2006; Karreman et al., 2009; Sartori et al., 2007).

Let us consider correlative microscopy to reveal the structure (or structures) of a nuclear compartment or domain. Ideally, such studies should start with a living cell with the intent to follow the fate of such a domain over a period of time. After the application of a fixation protocol suitable to preserve the in vivo 3D arrangement present immediately before fixation as best as possible, the same cells are reinvestigated sequentially by superresolution FM (with resolution beyond the Abbe limit) and finally by EM. The comparison of structural details seen in the living and the fixed state of the same cell allows an investigator to analyze the same nuclear domains of an individual cell. In addition, one can appreciate structural changes that may occur during the application of preembedding strategies to visualize distinct nuclear
constituents. Unfortunately, preparative procedures for FM observations often alter the fine nuclear structure, thus precluding further EM analysis. Consequently, the combination of living cell FM and subsequent EM investigation presently remains the best approach (Albiez et al., 2006). The optimal use of the concept of correlative microscopy for studies of nuclear architecture will in many cases require the generation of both FM and EM 3D images. While 3D reconstructions based on stacks of ultrathin sections are possible, one must keep in mind that it is quite demanding to make tens or even hundreds of consecutive sections without losing sections or section order. If one wishes to make serial sections from biological specimens much thicker than a single cell, for example, from entire mammalian preimplantation embryos, the number of consecutive sections required to obtain a full 3D reconstruction can quickly go up to thousands, while 3D reconstructions based on hundreds of consecutive light optical sections can routinely be obtained at the FM level. For a solution, one can combine ultramicrotomy with scanning EM (SEM) (Denk and Horstmann, 2004). Here, the surface of the specimen is imaged, an ultrathin section is removed by the ultramicrotome, the remaining surface is scanned again, and so forth (e.g., see Fig. 1.3). Whereas consecutive sections are the essential material for TEM, they play no role for imaging and are thrown away. This approach was applied for the first time, in combination with a selective preembedding staining of DNA, to reconstruct in 3D intranuclear distribution of chromatin and to quantitatively evaluate the part of the nuclear volume occupied by chromatin and by the chromatin-poor/-free interchromatin space (Rouquette et al., 2009). Alternatively, SEM can be combined with focused ion beam (FIB) milling (Knott et al., 2008) of the specimen. Here, an ion beam removes very thin layers of material (down to 10 nm), thus improving the \( z \) resolution. Schroeder-Reiter et al. (2009) used this approach for the first time to study the 3D ultrastructure of chromosomes. Compared with TEM, the resolution limit of SEM is lower but still considerably better than the resolution, which can presently be obtained with the most advanced superresolution FM microscopes overcoming the Abbe limit. The largest possible observation surface is much smaller with the FIB/SEM method (areas of about 20 \( \times \) 20 \( \mu \text{m}^2 \)) compared to the ultramicrotome/SEM method (up to several hundred micrometers). While both methods hold the promise to carry out 3D EM reconstructions from very large numbers of consecutive SEM images in a routinely applicable way, one should keep in mind that these two methods strictly require preembedding approaches with all the qualms concerning the typically less optimal structural preservation compared with postembedding approaches. Despite this limitation we expect that 3D EM reconstructions based on large series of consecutive SEM images can make important contributions to a future correlative microscopy, where single cells are followed from the living to the fixed state combining a wide range of FM and EM methods. In addition to SEM the use of TEM in cryoelectron microscopy can be considered for 3D studies using tomographical methods (Medalia et al., 2002), but the application seems for the moment limited to whole nuclei in the
studies of NPCs (Beck et al., 2004). Protocols proposing in vivo introduction of probes into the cells or directly into nuclei, suitable for both FM and EM analysis, may be another useful tool for future investigations enabling both FM and EM observations in successive steps on the same sample (Kireev et al., 2008).

6. Concluding Remarks

We present a brief historical account of light and electron microscopic studies on nuclear structure followed by an overview of current models of the functional nuclear architecture and of experimental support obtained with microscopic methods. We review present evidence for the intranuclear location of sites, where major nuclear functions such as DNA replication and repair, transcription, and RNA transport take place, focusing in particular on the topographical and structural implications of gene expression. In close relation to this point, we tried to evaluate the strength and the degree of reliability and reproducibility of different microscopic approaches that gave rise to such hypotheses and models.

Due to its superior resolution EM provided first evidence for a complex chromatin organization and—in combination with DNA-specific staining procedures—for a largely DNA-free interchromatin space carrying interchromatin granules and various nuclear bodies. In particular, EM was instrumental for revealing the PR as the major subcompartment for DNA replication, hnRNA synthesis, cotranscriptional splicing, and nucleotide excision DNA repair. FM combined with in situ hybridization (FISH) and immunocytochemistry provided direct evidence for CTs and their substructure, including chromatin domains with various patterns of histone modifications, as well as chromatin arrangements down to the positioning of individual genes.

We then point out problems and open questions in the field of functional nuclear architecture and discuss possibilities to answer them with emphasis on current limitations and new options of quantitative microscopic analyses. A great effort is being made in developing or perfecting light and electron microscopic techniques with the goal to follow cellular processes under the best experimental and methodological conditions. Compared with FM, EM has the decisive advantage of its highly superior resolution. The possibility to discriminate specific nuclear structures by multicolor approaches and the easiness to obtain light optical serial sections for 3D image reconstructions provide major advantages of CLSM. Spinning disk confocal microscopy is presently the most promising approach for 4D (space and time) studies of the dynamic nuclear organization in living cells. The introduction of in vivo markers for vital fluorescence microscopic analyses constitutes a major contribution and is closely related to advancements of molecular biological studies.
New types of FM have become available for cell imaging with a resolving power far beyond the Abbe limit of conventional light microscopy. This breakthrough has been accentuated by the new terms, light optical nanoscopy or superresolution microscopy, notwithstanding the fact of the still far superior resolution limit of EM. Staining of nuclear structures in fixed cells sometimes includes harsh preparative conditions, such as a DNA denaturing step prior to *in situ* hybridization. Structural damage caused by such procedures compromises the advantage of improved resolution. The applicability of light optical nanoscopy for live–cell studies is an urgently awaited further breakthrough, because such an approach, besides its impact for high resolution studies of nuclear dynamics, would allow rigorous tests how certain fixation and postfixation procedures alter fine structural features present in the living cell. New 3D EM approaches combining SEM either with ultramicrotomy or with FIB milling also show great promise for novel achievements.

Correlative microscopy consisting in successive observations by light microscopy *in vivo* followed by studies of the same cell after fixation with advanced light and electron microscopy is obviously the best approach to reveal the complex topography of major nuclear structures in 3D and 4D. It will help to capitalize on the particular strengths of each microscopic method and to avoid specific disadvantages. Major progress of our insight into the complex relationships between nuclear structure and function from dynamic higher order chromatin arrangements to the topography of protein machineries involved in transcription, splicing, DNA replication and repair, to topographical aspects of the nuclear import and export of macromolecules requires a system’s biology approach. For this purpose, methods of light and electron microscopy need to be combined with high-throughput methods for the mapping of DNA–DNA interactions in *cis* and *trans*, as well as DNA–protein and DNA–RNA interactions.

Microscopic methods are a powerful tool in the investigation of structure–function relationships in the cell nucleus. We hope that this review will provide a good overview of different approaches and will constitute a sort of guide to scientists who look for the right microscopic methods to be applied to their research problem.

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