Rise, fall and resurrection of chromosome territories: a historical perspective
Part II. Fall and resurrection of chromosome territories during the 1950s to 1980s.
Part III. Chromosome territories and the functional nuclear architecture: experiments and models from the 1990s to the present

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Part II of this historical review on the progress of nuclear architecture studies points out why the original hypothesis of chromosome territories from Carl Rabl and Theodor Boveri (described in part I) was abandoned during the 1950s and finally proven by compelling evidence forwarded by laser-uv-microbeam studies and *in situ* hybridization experiments. Part II also includes a section on the development of advanced light microscopic techniques breaking the classical Abbe limit written for readers with little knowledge about the present state of the theory of light microscopic resolution. These developments have made it possible to perform 3D distance measurements between genes or other specifically stained, nuclear structures with high precision at the nanometer scale. Moreover, it has become possible to record full images from fluorescent structures and perform quantitative measurements of their shapes and volumes at a level of resolution that until recently could only be achieved by electron microscopy. In part III we review the development of experiments and models of nuclear architecture since the 1990s. Emphasis is laid on the still strongly conflicting views about the basic principles of higher order chromatin organization. A concluding section explains what needs to be done to resolve these conflicts and to come closer to the final goal of all studies of the nuclear architecture, namely to understand the implications of nuclear architecture for nuclear functions.

Part II. Fall and resurrection of chromosome territories during the 1950s to 1980s.

In the late 19th and early 20th century Carl Rabl and Theodor Boveri proposed the first model of chromosome arrangements in the cell nucleus based on the hypothesis that interphase chromosomes occupy distinct territories (Cremer T., and C. Cremer, 2006b). This hypothesis was still supported during the 1940s (Cremer T., and C. Cremer, 2006b), but fell in disgrace during the 1950s to the 1970s. During this period the view became popular that only constitutive heterochromatin remains tightly condensed during interphase, whereas euchromatin strongly decondenses and intermingles (for reviews see (Comings, 1968; Vogel and Schroeder, 1974; Wischnitzer, 1973). The reasons for this change are not perfectly clear to us, but the fact that early electron microscopic studies failed to distinguish chromosome territories in ultra-thin nuclear sections was likely of major importance.

The concept of chromosome territories falls in disgrace

A review published in 1952 from Arthur W. Pollister on "Nucleoproteins of the nucleus" (Pollister, 1952) provides a telling example of this change of paradigm. Pollister presents "diagrams of two extremely different concepts of the relation of chromosomes to the nucleoprotein of the interphase nucleus." (Figure 1A,B) "In each case, to the cytologist the nucleus would appear very much the same; there would be a spherical nucleolus and a number of conspicuous heavily stained masses of chromatin (heterochromatin?). The latter are assumed to be regions where the chromatin thread is spiralized to a degree approaching that of the mitotic chromosomes. According to the view embodied in A, the
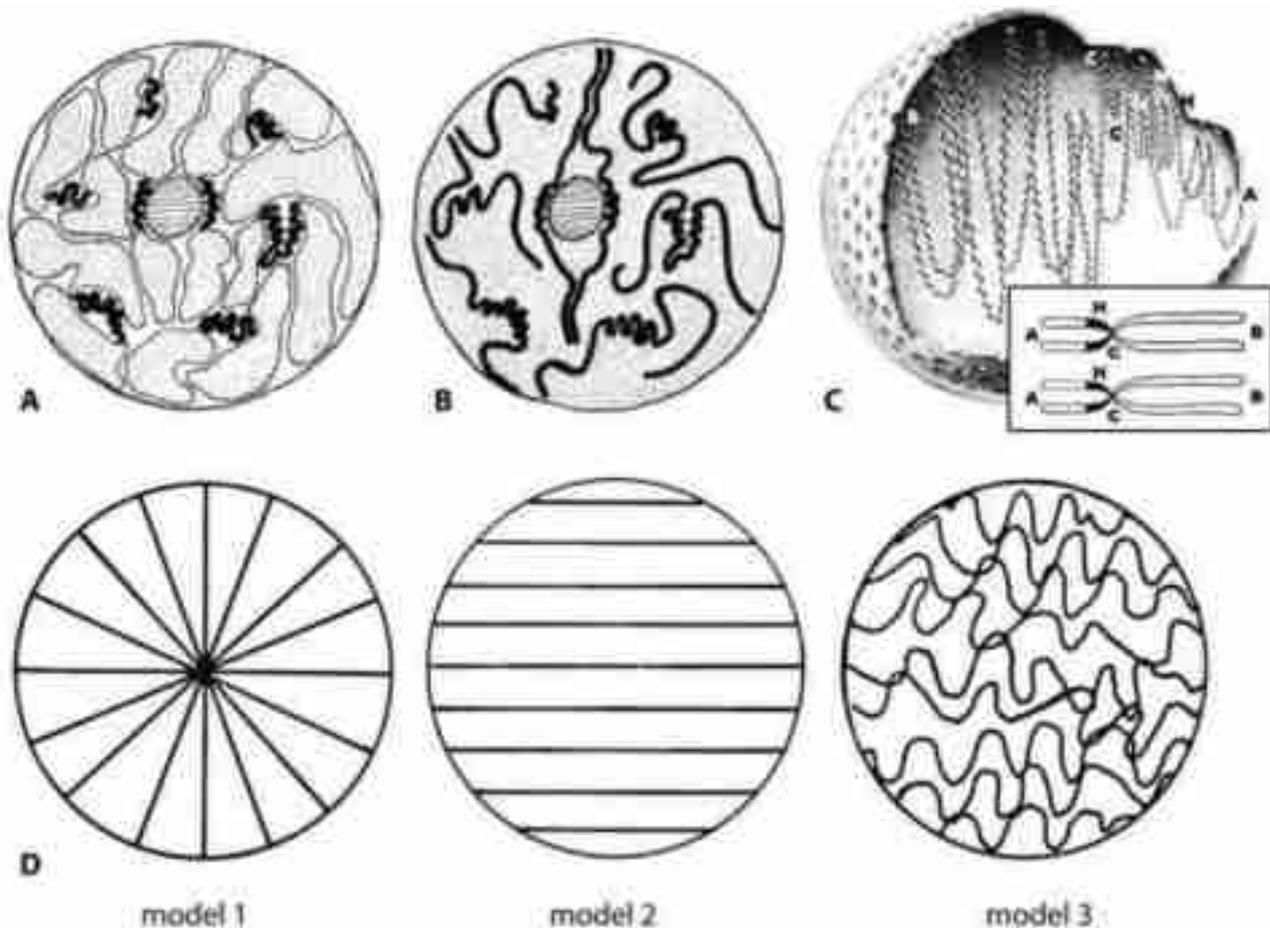
Figure 1. Hypothetical arrangements of interphase chromosomes and chromatin fibres published during the 1950s to 1980s
A, B) "Diagrams of two extremely different concepts of the relation of chromosomes to the nucleoprotein of the interphase nucleus" (Pollister, 1952). Pollister describes the two concepts as follows: "In each case, to the cytologist the nucleus would appear very much the same; there would be a spherical nucleolus and a number of conspicuous heavily stained masses of chromatin (heterochromatin?). The latter are assumed to be regions where the chromatin thread is spiralized to a degree approaching that of the mitotic chromosomes. According to the view embodied in A, the non heterochromatic parts of the chromosomes are swollen masses, which together almost completely fill the nucleus; thus, except for the nucleolus, the nuclear contents are intrachromosomal. In B the chromosomes are slender structures throughout, they occupy but a small part of the nuclear volume, and the main bulk of the nucleus is a formless nuclear sap, in which the chromosome threads are floating." C) Model of interphase chromosome arrangements (adapted from (Comings, 1968) with permission). Comings model shows chromatin threads of two spatially associated, homologous interphase chromosomes expanding throughout the major part of the nuclear space. Attachment sites are restricted to the nuclear envelope (for further details see text). D) Hypothetical arrangements of 30 nm chromatin threads in the human interphase nucleus (reprinted from (Vogel and Schroeder, 1974) with permission; for further details see text).

non heterochromatic parts of the chromosomes are swollen masses, which together almost completely fill the nucleus; thus, except for the nucleolus, the nuclear contents are intrachromosomal. In B the chromosomes are slender structures throughout, they occupy but a small part of the nuclear volume, and the main bulk of the nucleus is a formless nuclear sap, in which the chromosome threads are floating." Obviously, according to model A a notable interchromosome space may or may not be detectable depending on the extent to which interphase chromosomes swell. For Pollister such an interchromosomal space, if at all detectable in some cell types, did not seem to have any particular functional importance. He apparently preferred the concept "that the nucleus is a ball of long, thread-like chromosomes, more or less entangled – like a ball of yarn with many discontinuous pieces". While he felt that "the actual structure of the interphase nucleus may possibly range anywhere between A and B in different cell types, he was fully aware of the lack of compelling evidence in favor of one or other model of nuclear architecture and wisely emphasized: "Only by a combination of chemical, cytological, and cytochemical evidence can we hope eventually to understand the relationship between

Figure 2. Scheme of a liver cell nucleus from Monneron and Bernhard (1969). This scheme (reprinted from (Monneron and Bernhard, 1969) with permission) shows the distribution of major structural components, which had been detected in electron microscopic studies at the end of the 1960s: chr: chromatin; nu: nucleolus; ig: interchromatin granules: pg: perichromatin granules; cb: coiled bodies; arrows point to perichromatin fibrils. Note that perichromatin fibrils are depicted at the periphery of chromatin clumps (indicating higher order chromatin structures above the level of 30 nm chromatin fibers). Other fibrils are seen in addition to interchromatin granules and coiled bodies within an extended interchromatin space, which is apparently free of chromatin fibres. Monneron and Bernhard report that "a rather constant pattern of the distribution of nuclear substructures is observed in all cells examined. These include "the condensed wall [of dense chromatin] along the nuclear membrane, the nucleolus-associated chromatin and the clumps of chromatin dispersed in the nucleoplasm." Monneron and Bernhard avoided to speculate about the structure of such clumps of chromatin, but it is important to note that their scheme emphasizes only such clumps and lacks any indication of a predominant fraction of thin and and thick chromatin fibres expanding in the interchromatin space. Taking into account that their scheme of the nuclear architecture is based on transmission electronic microscopy of ultrathin nuclear sections, it is in complete accordance with the concept of chromosome territories. Isolated chromatin clumps seen in such sections participate in a higher order chromatin network above the level of 30 nm thick chromatin fibres, which connects neighboring CTs with each other and is pervaded by an interchromatin compartment (compare Figures 23A, 24) (for experimental evidence see (Albiez et al., 2006).

conspicious mitotic chromosomes and the interphase nucleus" This remark could still be made today in any state-of-the-art review of nuclear architecture.

In 1968 Comings (Comings, 1968) published a scheme of a human nucleus (Figure 1C), which shows chromatin threads of two homologous interphase chromosomes expanding throughout the major part of the nuclear space. He proposed 1. that all replicons are attached to the nuclear membrane; 2. that these attachment sites are widely separated in euchromatin but crowded together in heterochromatin; 3. that attachment sites for homologous chromosomes were located in proximity resulting in homologous association. Although Comings cited Boveri's "classic studies of Ascaris in 1909" (Boveri, 1909), he did not mention Boveri's concept of chromosome territories (for details see the first part of this review (Cremer T. and Cremer C., 2006b)).

During the 1970s a wealth of new data on the structure of interphase chromatin was published, including the discovery of the nucleosomes (Kornberg and Thomas, 1974; Olins and Olins, 1974) for reviews see (Felsenfeld and McGhee, 1986; Olins and Olins, 2003). Available electron microscopic evidence strengthened the idea that at the onset of interphase mitotic chromosomes unravel into extended chromatin fibres, which mingle in the nucleoplasm (DuPraw and Bahr, 1969). In a review on the submicroscopic morphology of the interphase nucleus, Wischnitzer (1973) claimed that electron microscopic studies had "established that discrete interphase chromosomes are absent." In spite of the failure to distinguish individual CTs, the contribution of electron microscopic studies to a better understanding of the nuclear architecture can hardly be overestimated. An in-depth review of these studies is beyond the scope of this article, but we wish to emphasize, if only as an example, the seminal contributions of Wilhelm Bernhard (1920-1978) and his colleagues to the field. In landmark papers on the fine structural organization of the cell nucleus, he and his co-workers described the ultrastructure and nuclear topography of distinct nuclear constituents, such as interchromatin- and perichromatin granules, perichromatin fibrils and coiled bodies (Monneron and Bernhard, 1969) (Figure 2). The latter are now preferentially termed Cajal bodies in honour of Santiago Ramón y Cajal (1852-1934), who first described them in 1903 as
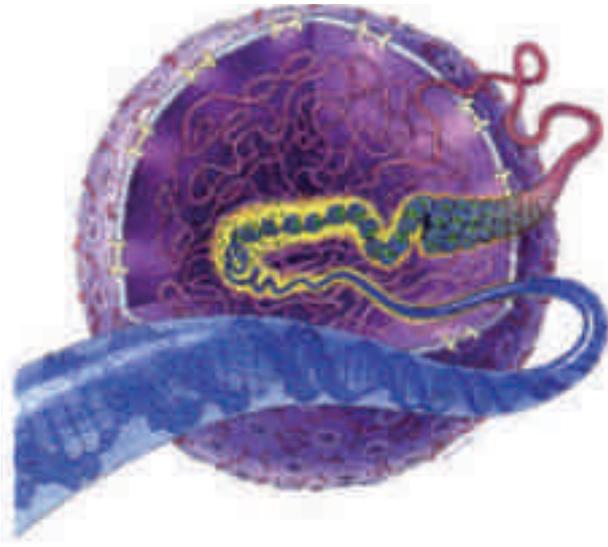
Figure 3. Typical undergraduate textbook scheme of the nuclear architecture from the 1990s. This scheme serves as the frontis piece of Stephen L. Wolfe's excellent textbook "Molecular and Cellular Biology" published in 1993 (reprinted with permission from Wadsworth Publishing Company, Belmont, California). It emphasizes intermingling of giant 30 nm thick chromatin loops intermingling in the nuclear sap like noodles in a noodle soup. Undergraduate students are alluded to the importance of detailed knowledge about the DNA structure, nucleosomes, thin and thick chromatin fibres. At the same time they also receive the impression that a functionally important higher order nuclear architecture does not exist and hence does not present an unsolved problem of cell biology. This view still prevails in some textbooks despite the compelling evidence for chromosome territories and a compartmentalized nuclear architecture. Textbooks may naively be considered as sources of sound scientific knowledge. For a historian of science, however, they provide rich sources to document blind spots of research at a given time period. For a beginner it is impossible to distinguish in such schemes those features, which are strongly supported by experimental evidence, from others, which document a view prevailing in the scientific community, even if the evidence for it is meagre or non-existent at all.

nucleolar accessory bodies of neuronal cell nuclei (Ogg and Lamond, 2002). Another seminal paper from Bernhard's group provided the first evidence for the formation of nascent RNA in the perichromatin region (Fakan and Bernhard, 1971). This publication was selected in 2001 by the American Society for Cell Biology for a book "Landmark Papers in Cell Biology" (eds. J.G. Gall & J.R. McIntosh) published by Cold Spring Harbor Laboratory Press. Wilhelm Bernhard and his colleagues have put these structures on the map of the nuclear landscape like geographical landmarks of a newly discovered continent.

Based on Comings' seminal paper from 1968 Vogel and Schroeder (Vogel and Schroeder, 1974) published their considerations on the internal order of the human interphase nucleus. They assumed that most chromatin except for some heterochromatic regions was present in chromatin threads with a

packaging ratio of 1:30 and that these threads were solely fixed at nuclear pores. Based on these assumptions, they discussed three models of how 30 nm chromatin fibres might be arranged (Figure 1D). These models exemplify the extreme cases of a conceptual trend to emphasize the decondensation of interphase chromosomes into very long 30 nm fibres or loops expanding throughout the whole nuclear space. This trend was driven by intellectual preference, while new and compelling experimental evidence for this concept was lacking. In their model 1 Vogel and Schroeder assumed equally sized segments of a contiguous 30 nm fibre fixed at opposite nuclear pores and expanding throughout the centre of the nucleus. In model 2 segments run parallel and vary in size. Model 3 differs from model 2 by the assumption that chromatin threads are not tightly stretched but arranged more loosely. Taking into account that a diploid human nucleus contains ca. 6×10 exp. 9 nucleotide pairs Vogel and Schroeder calculated the number of predicted fixation points at the nuclear envelope for their three models and found that this number "corresponds almost too well" with estimates of about 8000 nuclear pores of a human lymphocyte nucleus estimated in electron microscopic studies. While the finding that a given model is consistent with numbers obtained by experiment typically creates a moment of joy for the inventors of a new model, such a coincidence neither proves the model's validity nor the validity of the supporting experimental data. Encouraged by this coincidence Vogel and Schroeder, however, felt that their models 2 and 3, respectively, provided a reasonable representation of the true organization of chromatin threads. "The real value [for the number of fixation points] might be somewhere between the values calculated by models 2 and 3." Model 1 was abandoned, because its prediction of a higher chromatin density in the nuclear interior was not supported by electron microscopic studies, whereas both models 2 and 3 were consistent with a fairly equal distribution of chromatin throughout the nuclear space.

Most molecular biologists with an interest in nuclear biology seemed to be content at this time with the assumption that chromosome arrangements in the cell nucleus are comparable to a ball of yarn composed of the random arrangements of many discontinuous pieces, an impression reinforced to students by schemes of the nucleus in cell biology textbooks (Figure 3). A reader might think

that Comings, as well as Vogel and Schroeder were advocates of a random intermingling of chromatin threads. Yet, this would be a profound misunderstanding. In contrast, these authors belonged to the minority of scientists, also including Gunter Blobel (Blobel, 1985) and a few others, who kept an interest in the problem of nuclear architecture for the very reason that they expected functionally relevant, cell type specific higher order chromatin arrangements, whatever this functional relevance might be. However, "any degree of order [with respect to the arrangements of chromatin threads] would be hidden by the very mass of the chromatin itself" (Comings, 1968).

It is likely not by coincidence that interest in studies of higher order chromatin arrangements decreased at a time, when molecular biology started to dominate biological research as the seemingly one and only way to define and answer big biological questions, marginalizing all other approaches. Whereas the importance to sequence DNA and to define essential transcription factors was obvious, the packaging of DNA with histones seemed to be of less functional interest and studies of interphase chromosome arrangements appeared as a barren field. What kind of a major insight should one still expect from light microscopical studies? Numerous studies had been performed to analyze the arrangements of chromosomes in metaphase spreads of various species (for review see (Comings, 1980). The results, however, were contradictory and did not help to encourage a wide community of researchers to speculate about a functional meaning of higher order chromatin organization in the cell nucleus, not to speak of the development of experimental approaches to test such speculations. Factors as different chromosome size and DNA sequence, different protein content and compaction, as well as replication timing could all contribute to some deviation of chromosome arrangements from random expectation, yet non-random arrangements of this kind did not make a strong point per se to expect any important implications of higher order chromatin arrangements on nuclear functions.

The state of insecure knowledge and insecure opinions at the mid 1980s about higher order chromatin arrangements is reflected by a review from 1987 (Cremer T., Emmerich P. *et al.,* 1987) with the title "Does a cell type specific arrangement of chromatin exist in mammalian cell nuclei? Arguments of non-believers, agnostics and believ-

ers." The review started with the views of the scientific infidels: "Except for a Rabl orientation observed in nuclei of some cell types as a consequence of the anaphase-telophase movements of chromosomes, their arrangements turned out to be (largely) random. It cannot be expected that additional and methodologically increasingly elaborate studies will lead to principally new insights, apt to increase substantially our understanding of the structure and function of the mammalian genome." In contrast, "a small group of believers postulated a high degree of a functionally important, cell type specific order of chromosomes in the cell nucleus." Günter Blobel's "gene gating hypothesis" from 1985 served as an example (Blobel, 1985). Blobel argued "that the genome of a higher eukaryotic organism is organized into a number of distinct three-dimensional (3-D) structures, each characteristic for a given differentiated state. These discrete 3D-structures are envisioned to develop in a hierarchical and largely irreversible manner from an omnipotent 3-D structure of the zygotic genome." We were encouraged at lot by "believers", who dared to propose functionally extremely interesting models, if true, but were rather inclined to take the position of "agnostics", summarized as follows: "Published studies so far regarding the topography of chromosomes in the cell nucleus are unsatisfactory, contradictory and all together insufficient to either exclude or positively prove a conceivable functional importance of chromosome topography." ... "A pronounced variability in the arrangement of certain chromosomes studied so far in certain cell types cultured *in vitro* does by no means eliminate the possibility that a functionally important and cell type specific topography exists with respect to other chromosomes or other criteria (see below), which we have not discovered so far for mental reasons (lack of an appropriate theory) and because of methodological shortcomings. " ... "To solve the problem of chromosome topography in a comprehensive way, it is necessary to perform comparative studies in diversely differentiated cell types of an organism, as well as comparative studies of the same distinct cell types of different species. Questions, which need to be answered, reflect
a) the structure and extension of individual chromosomes during interphase and mitosis;
b) specific and non-specific attachment sites of chromosomes at the nuclear envelope and a nuclear matrix, respectively;
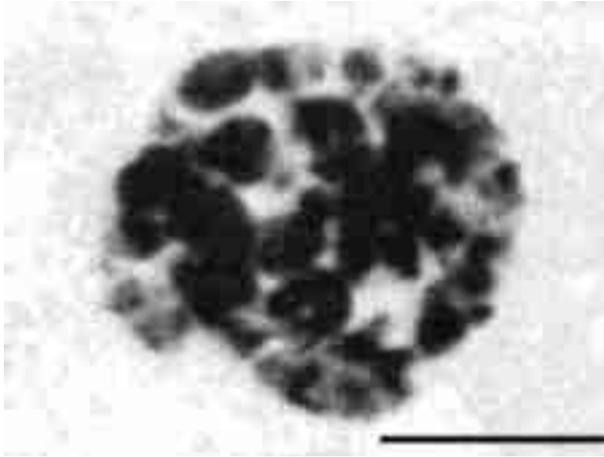
Figure 4. Evidence for chromosome territories provided by Stephen B. Stack, David B. Brown and William C. Dewey (1977). Nucleus of a Chinese hamster cell (CHO line) in mid G1 shows clumps of condensed chromatin likely representing chromosome territories (reprinted with permission from (Stack et al., 1977). Trypsinized cells were fixed with acetic acid/ ethanol (1:3) and squashed immediately thereafter in 50% acetic acid. Squashed, air dried cells were briefly submerged in a solution of 0.02 N NaOH and 0.114M NaCl at room temperature and their chromatin finally coloured with Giemsa stain. Stack et al. propose that "the successive treatments cause shrinkage and swelling of chromatin that results in its being visibly separated into distinct domains or clumps that correspond to individual chromosomes" and further that "sodium-hydroxide-treated G1 nuclei also indicate the nucleoplasm consists largely of elongate, swollen chromosomes". Bar corresponds to 10 μm.

c) the possibility of a specific orientation and folding of individual chromosomes;
d) chromatin movements during the cell cycle and during the terminal differentiation of postmitotic cells;
e) the spatial arrangement of active and silent genes within individual interphase chromosomes and finally;
f) the relative arrangements of homologous and non-homologous chromosomes in the cell nucleus.

When studying the examples cited from the literature and from pertinent reviews (Comings, 1980; Hubert and Bourgeois, 1986), the critical reader will find that definitive answers are lacking for all these questions."

Resurrection of chromosome territories: final proof of an old concept

During the early 1970s the concept of chromosome territories was still neither proven nor disproven by compelling evidence. In 1977 Stephen M. Stack, David B. Brown and William C. Dewey (Stack *et al.,* 1977) published a seminal paper defending the existence of chromosome territories. They fixed cells with acetic acid/methanol (1:3)

and squashed them immediately in 50% acetic acid. Air dried cells were subjected to a combination of NaOH treatment, dehydration in ethanol and staining with Giemsa. This treatment yielded nuclei containing "what seem to be distinct interphase chromosomes throughout the cell cycle of *Allium cepa* and Chinese hamster (CHO) nuclei." (Figure 4) "Chromosomes appear never to decondense to the point that they loose their 3-dimensional integrity, but remain in distinct domains throughout interphase." Stack and co-authors claimed that this observation not only was "generally in agreement with many older light-microscopic descriptions of interphase nuclei and chromosomes" but considered it - in contrast to Wischnitzer's above cited dictum from 1973 - "as being compatible with and complementary to descriptions of interphase nuclei based on electron microscopy". Our recent finding that CTs form a contiguous 3D chromatin network together with interchromatin compartment network (Albiez *et al.,* 2006) (*see below*) is fully compatible with the ultrastructural scheme of the nuclear architecture provided by Monneron and Bernhard in 1969 (Figure 3). Stack *et al.* (1978) further pointed out: "If one assumes the nucleus is composed essentially of swollen chromosomes between which there is ordinarily no space other than that occupied by nucleoli, the 10-30 nm fibres may intermingle to some extent on the borders of chromosomes. This would blur the boundaries of chromosomes at the levels of both light and electron microscopy." ... "Attachment of chromosome fibres to the nuclear envelope probably is not the sole basis of maintaining the structure of chromosomes throughout interphase, because many of the chromosomes of *A. cepha* condense at prophase without apparent association with the nuclear envelope." If so, what mechanism could provide interior fixation points to maintain the chromosome territory architecture? Stack *et al.* cite a "suggestion of (Comings and Okada, 1976) that chromosomes condense in association with a protein network called the 'nuclear matrix' (Berezney and Coffey, 1976), that includes not only the nuclear-envelope-pore complex but a fibrous network throughout the nucleoplasm. The precise relationship between this fibrous network and individual chromosomes is not yet clear."
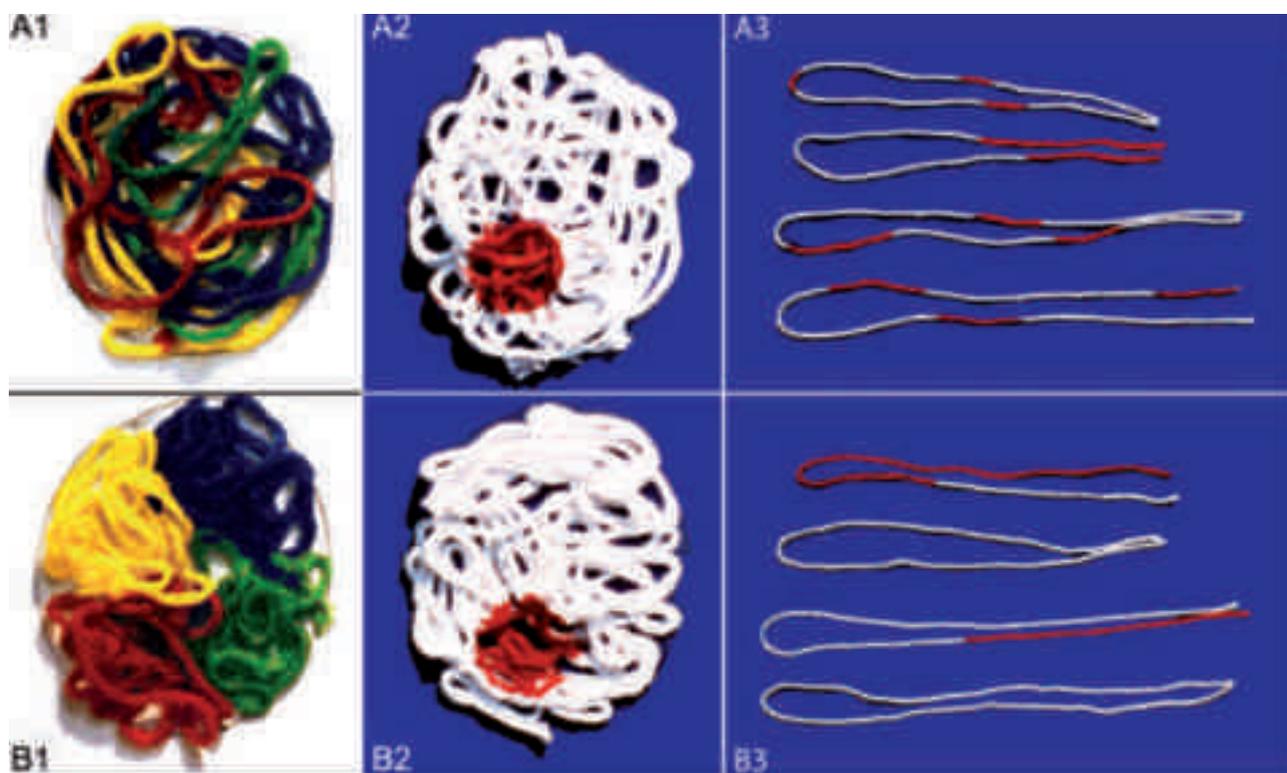
Figure 5. Experimental rationale of microirradiation experiments. (T. Cremer and C. Cremer, unpublished scheme from 1982, compare Figure 3 in Cremer T. , Cremer C. et al. 1982). The higher order chromatin arrangement in the interphase nucleus is compared with a coil composed of a number of threads (chromosomes). The examples shown do not refer to an actual chromosome arrangement of any particular cell type. For further details see text. A1) The threads are extended and intermingled B1) Each thread forms a distinct territory. A2 and B2) The arrangements of threads shown in A1 and A2 cannot be distinguished, when all threads bear the same colour. A small part of the coil is marked by red colour. A3 and B3) Threads show red marks after disassembling of the two coils. The distribution of these marks shows the regions of the threads located together in the two coils.

Studies of the nuclear architecture with laser UV-microbeam experiments

Experimental rationale of laser microbeam experiments

In 1968 – being students of medicine and physics, respectively - we became excited by the possibilities of lasers as a research tool in cell and developmental biology (Berns *et al.,* 1969a; Berns *et al.,* 1969b) for a comprehensive review of the emerging use of lasers as tools for the micromanipulation of individual cells at this time see (Bereiter-Hahn, 1972a; Bereiter-Hahn, 1972b). This excitement prompted us to write a research proposal to develop an UV-laser microbeam (Cremer C. and Cremer T., 1969). We outlined a series of possible applications including the use of such an instrument to elucidate interphase chromosome arrangements: "Does an ordered arrangement of interphase chromosomes exist, which changes regularly with different states of function? Could it be that genes, which functionally belong together, are also positioned closely together, even if they are located on different chromosomes? If so, a method, which allows the isolated damage of small nuclear areas, might help to establish statistically significant "functional" linkage groups, which need not always be consistent with gene maps established by genetic experiments. A spherical nucleus with a radius of 5 μm has a volume of about 500 μm³...If we assume that laser light could be focused down to a cube with a side length of 0.2 μm, we would have 60.000 different possibilities at our disposal for selective destructions in such a nucleus."

Figure 5 points out our experimental rationale to study higher order chromatin arrangements (Cremer T. , Cremer C. et al., 1982a). We compared the interphase nucleus and its chromosomes with a coil composed of a number of threads. Each thread may extend throughout the whole coil (case A; Figure 5A1) or form a distinct territory (case B; Figure 5B1). When all threads are of the same colour (white in our example), it is impossible to recognize the arrangements of each individual
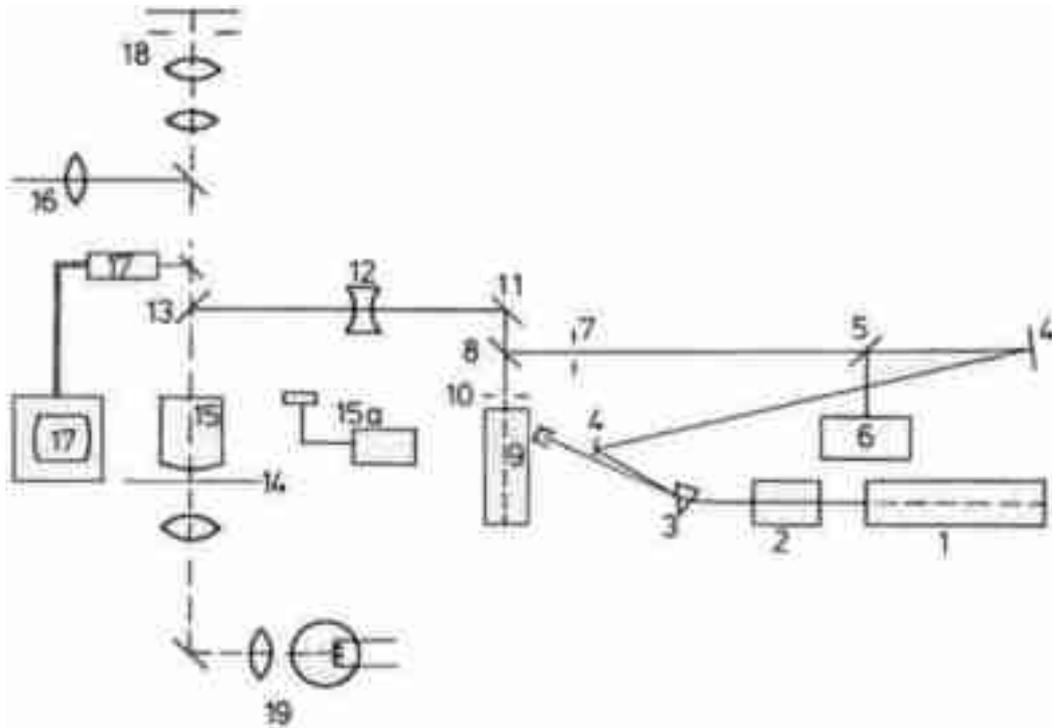
Figure 6. Diagram of laser-UV-microbeam (reprinted from (Cremer C., Zorn C., Cremer, T. *et al.,* 1974) with permission) 1) coherent wave argon ion laser (λ = 514 nm); 2) UV-generator for the generation of coherent UV-light (λ = 257 nm); 3) separation prism; 4) beam deflection mirror; 5) beam splitter; 6) UV-detector system; 7) shutter; 8) selecting mirror; 9) He-Ne Laser produces a visible pilot beam, co-aligned with the UV-beam; 10) shutter; 11) adjustable mirror; 12) dispersing lens; 13) selective mirror able to deflect the UV-beam, while transmitting the visible light; 14) object plane; 15) Zeiss ultrafluar microscope objective 100x; 15a) photodiode for a transient replacement of the microscope objective to measure incident UV-light; 16) ocular system; 17) TV-camera; 18) camera system; 19) illumination system.

thread in the two coils. Red colouring of a small area of the coil (Figure 5A2, B2) and subsequent separation the individual threads (Figure 5A3, B3) provides a way to analyse, which segments of the threads were located in spatial proximity within a given coil. In case A the red marks are scattered over all threads emphasizing their non-territorial organization in the coil (Figure 5A3) In case B the territorial organization is indicated by long, red coloured segments detected on two threads, whereas the two other threads remain entirely white (Figure 5B3). Our example shows only four threads. In coils with much larger numbers of threads, the difference between case A and B would be even more pronounced. In case of a territorial organization only a small minority of the threads should bear red marks. For the realization of this rationale it was necessary to achieve three goals: 1. modification of DNA restricted to a small part of the nucleus in living cells; 2. detection of the modified DNA *in situ*; 3. tracing of individual, microirradiated cells from interphase to mitosis and detection of the modified DNA on metaphase chromosomes.

Construction of laser-UV-microirradiation instrument

To achieve goal 1 we built an instrument that allowed laser-UV-microirradiation experiments of living cells (Figure 6) (Cremer C. *et al.,* 1974) and developed protocols to detect microirradiated DNA *in situ*. Frequency doubling of the 514 nm line of a continuous wave argon ion laser yielded coherent UV-light with λ = 257 nm. This wavelength is sufficiently close to the absorption peak of DNA at λ = 260 nm. The UV-beam was typically focused to a minimum spot diameter of ~0.5 μm in the object plane of a microscope equipped with quartz lenses. When the beam was focused somewhat above the focal plane, it was also possible to microirradiate a larger part of the nucleus or even most of the nuclear area. In this way a given incident UV-dose could be distributed in different ways (*see below*). Living cells were maintained at the microscope stage in a temperature controlled tissue chamber with a quartz window to allow UV-microirradiation of cells.
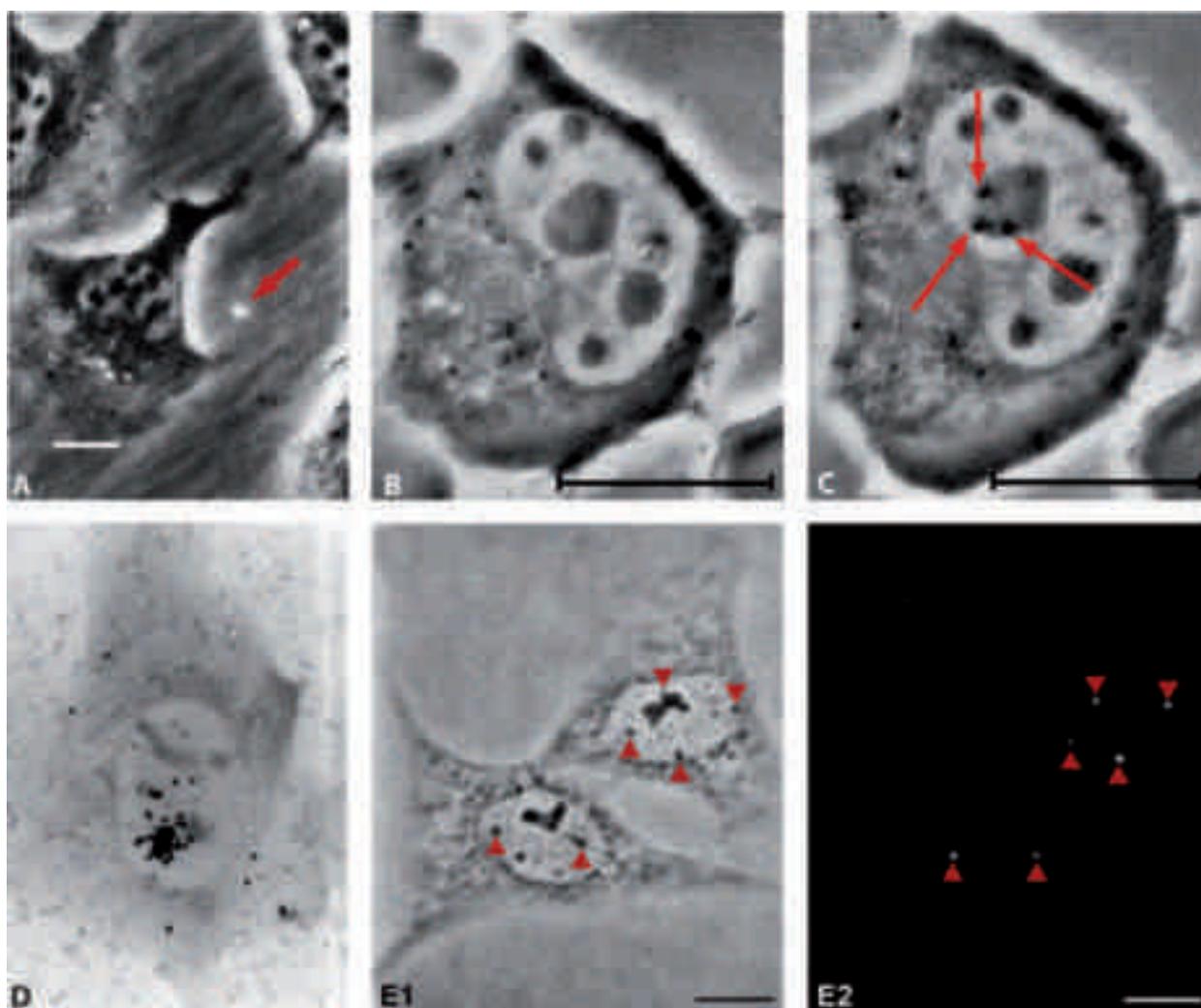
Figure 7. Laser-UV-microirradiation of Chinese hamster cell nuclei. A) Phase contrast micrograph of a living Chinese hamster cell (line V79). The laser UV beam, which was focused onto the substratum beside this cell, induced a fluorescent spot (arrow). B and C) Living Chinese hamster cell (V79 line) before (B) and after (C) microirradiation of the nucleolar edge at three sites. Arrows indicate three visible lesions (reprinted from (Cremer C. et al., 1974) with permission). D) Autoradiograph of the nucleus of a diploid Chinese hamster fibroblastoid cell microirradiated in G1. A cluster of silver grains marks the site of nuclear microirradiation (reprinted from Zorn et al., 1979) with permission). Living, microirradiated cells were incubated in medium with [³H] thymidine for 2 hours to detect unscheduled DNA synthesis, which occurred during excision repair of microirradiated DNA. Thereafter fixation and autoradiography was performed. E) Two nuclei of living Chinese hamster cells (line M3-1) were microirradiated at two and four sites, respectively. In phase contrast microscopy (left) the sites of microirradiaton are detectable as small dark spots. Microirradiated DNA contained at these sites was visualized by indirect immunofluorescence with primary antibody specific for UV-irradiated DNA (for further details see (Cremer C., Cremer T. et al., 1983). Bars represent 10 µm.

Detection of UV-microirradiated DNA *in situ*

For our UV-microbeam we chose diploid Chinese hamster cells, which carry a diploid set of 22 chromosomes, as well as cell lines with slightly higher modal chromosome numbers. When the UV-microbeam was focussed on the growth surface beside a living cell, it induced visible fluorescence (Figure 7A). A reticle (cross-hair) in the ocular was used for precise alignment of the microbeam to a site of the cell chosen for microirradiation. Following microirradiation of the nuclear interior, e.g. at the edge of a nucleolus, we observed visible

lesions (Figure 7B and C). These lesions, however, were only transiently visible. Two approaches were developed to visualize UV-microirradiated DNA *in situ*: 1. visualization of unscheduled DNA synthesis (UDS) during excision repair of DNA photolesions and 2. immunostaining of microirradiated DNA with specific DNA antibodies.

First, we microirradiated nuclei of living Chinese hamster cells in G1 at a single site, pulse-labelled them with ³H-thymidine prior to fixation. This radioactive DNA precursor carrying UV-induced DNA photolesions, in particular pyrimidine dimers,

incorporated and was detected by autoradiography (Figure 7D) (Cremer C. *et al.,* 1981a; Zorn *et al.,* 1979). A quantitative analysis indicated that silver grain densities significantly over background levels were only observed at the microirradiated nuclear area, while no increase of background levels was observed at remote nuclear sites compared to non-irradiated control nuclei. This result, as well as the fact that microirradiation of cytoplasm beside nuclei did not induce UDS detectable *in situ*, argued that global genome damage due to UV-stray light was negligible.

Second, we performed immunolabelling of microirradiated DNA *in situ* with antibodies that bind specifically to UV irradiated DNA (Cremer C., Cremer T. *et al.,* 1980a; Cremer T., Baumann H. *et al.,* 1984a; Hens *et al.,* 1983). (Figure 7E).

Evidence for chromosome territories obtained by the analysis of chromosomal damage in microirradiated cells

Following microirradiation of small parts of nuclei (about 5% of the total nuclear area) from diploid, fibroblastoid Chinese hamster cells established from lung tissue, we followed single, microirradiated cells from interphase to mitosis, performed metaphase chromosome spreads *in situ* and analyzed these spreads for chromosome aberrations (Hens *et al.,* 1983). We expected that these aberrant chromosomes would represent chromosomes contributing to the microirradiated nuclear area. In spite of a high energy density of the microbeam at its focal site (several thousand ergs/mm^2; 1 erg = 10^{-7} J), the yield of metaphase spreads with damaged chromosomes was low. In an effort to enhance the yield of chromosome aberrations we combined microirradiation with caffein post-treatment (1-2 mM). Caffein interferes with post-replication repair and increased the yield of metaphase spreads with damaged chromosomes in cells with microirradiated nuclei drastically, but did not induce chromsome damage in unirradiated cells or in cells microirradiated in the cytoplasm. Metaphase spreads were classified into three categories: category I comprised spreads with apparently intact chromosomes, category II spreads with a few structurally aberrant chromosomes beside a majority of intact chromosomes (Figure 8A,B), whereas in spreads of category III the entire chromosome complement appeared shattered or even pulverized (Zorn *et al.,* 1976) (Figure 8C,D,E). In later studies we referred

to spreads of category II as partial chromosome shattering (PCS) and of category III as general chromosome shattering (GCS) (Cremer T. *et al.,* 1982b). The observation of cells with PCS and GCS side by side was unexpected. At face value PCS was consistent with the hypothesis of chromosome territories, while GCS was indicative of nuclei with pronounced intermingling of interphase chromosomes. Experiments, where premature chromosome condensation in interphase nuclei was induced by fusion of interphase with mitotic cells, had suggested that the length of prematurely condensed chromosomes varied during the cell cycle. Their length increased during G1, while their induction in S-phase cells revealed an apparently pulverized appearance (Rao *et al.,* 1977). This finding suggested the possibility of a non-territorial organization of interphase chromosomes during S-phase and a territorial organization during G1 and G2.

To demonstrate convincingly that shattered chromosomes in cells with PCS represented CTs directly hit by the UV-microbeam, it was necessary to show that DNA-photolesions in PCS cells coincide with the area of shattered chromosomes. Immunostaining of microirradiated DNA (see above) in metaphase spreads with PCS demonstrated that the UV-microirradiated DNA was restricted to the area of shattered or pulverized chromosomes (Figure 9A,B). In cells with GCS, immunostaining was focally restricted to a small fraction of the shattered chromosome complement emphasizing an indirect effect of microirradiation (Figure 9C,D). This result ruled out the assumption that metaphase spreads with GCS were derived from interphase nuclei with a non-territorial organization of interphase chromosomes. Instead it argued for indirect effects of microirradiated DNA on chromatin located remote from the microirradiated nuclear area (*see below*). In a subsequent study we used PCS as an endpoint to analyze CT arrangements in nuclei of fibroblastoid, diploid Chinese hamster cells. In a total of about 13.000 living cells nuclear areas with diameters of about 1-2 μm were microirradiated. Metaphase spreads with PCS and GCS, respectively, were prepared after a post-microirradiation incubation period of 7-9 hours in medium with 0.5 mM caffein. In 60 metaphase spreads with PCS the average number of damaged chromosomes was 4.5 (range 1-11). In karyotypes of cells with PCS we noted a considerable variation with regard to simultaneously shat-
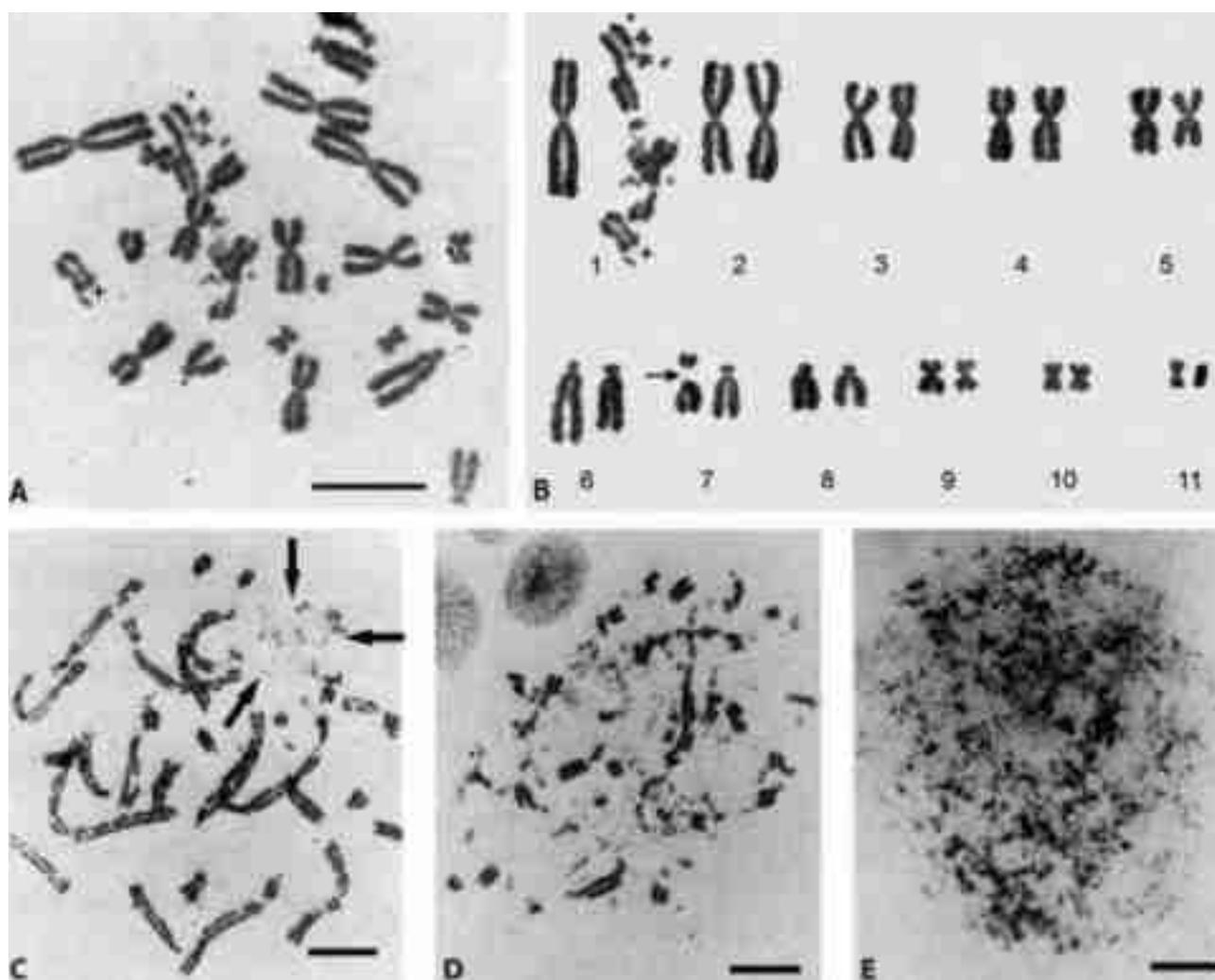
Figure 8. Metaphase spreads with damaged chromosomes obtained after laser-UV-microirradiation of nuclei in living Chinese hamster cells. Nuclei in living Chinese hamster cells were microirradiated (λ = 257 nm) at a single nuclear sites comprising about 5% of the total nuclear area. Microirradiated cells were followed to the next mitosis (about 3 to 15 hours) in medium with 1 mM caffeine. A and B) Metaphase spread (A) and the corresponding karyogram (B) from a diploid, fibroblastoid Chinese hamster cell reveal a shattered chromosome 1 and a break in a chromosome 7 surrounded by intact chromosomes (reprinted from Zorn *et al.*, 1976 with permission). C) Metaphase spread from a diploid fibroblastoid Chinese hamster cell shows an area of pulverized chromosomes (arrows) surrounded by chromosomes with chromatid gaps or breaks (reprinted from (Zorn et al., 1976) with permission). D) Mitotic V79 cell showing fragmentation and pulverization of the whole chromosome complement (reprinted with permission from Cremer C., Cremer, T. *et al.*, 1981a. E) Mitotic V79 cell with a pulverized chromosome complement (reprinted from (Cremer C., Cremer, T. *et al.*, 1981a) with permission). Bars correspond to 10 µm.

tered chromosomes indicating variable arrangements of CTs. Consistent with the expectation that large chromosomes should occupy larger territories than small ones, we found a positive correlation ($p<0.05$) between the relative DNA content of individual chromosomes and the frequency with which they participated in PCS (Cremer T., Cremer C. *et al.,* 1982b). Joint damage of homologous autosomes was a rare event in comparison with the frequencies of joint damage of non-homologues chromosomes of similar size. For the cell strain studied by us this results ruled out Coming's hypothesis of a close spatial association of homologous CTs.

Evidence for chromosome territories obtained by the analysis of unscheduled DNA synthesis in UV-microirraded cells

As an alternative, much more compelling, but also much more elaborated approach we made use of unscheduled DNA synthesis in microirradiated chromatin to study interphase chromosome arrangements. We microirradiated Chinese hamster cell nuclei in G1 at a single site, pulse-labelled them with ³H-thymidine and allowed them to continue the cell cycle until a reasonable fraction entered the next mitosis (Cremer T., Cremer C. *et al.,* 1982a; Zorn *et al.,* 1979). Metaphase spreads prepared *in*
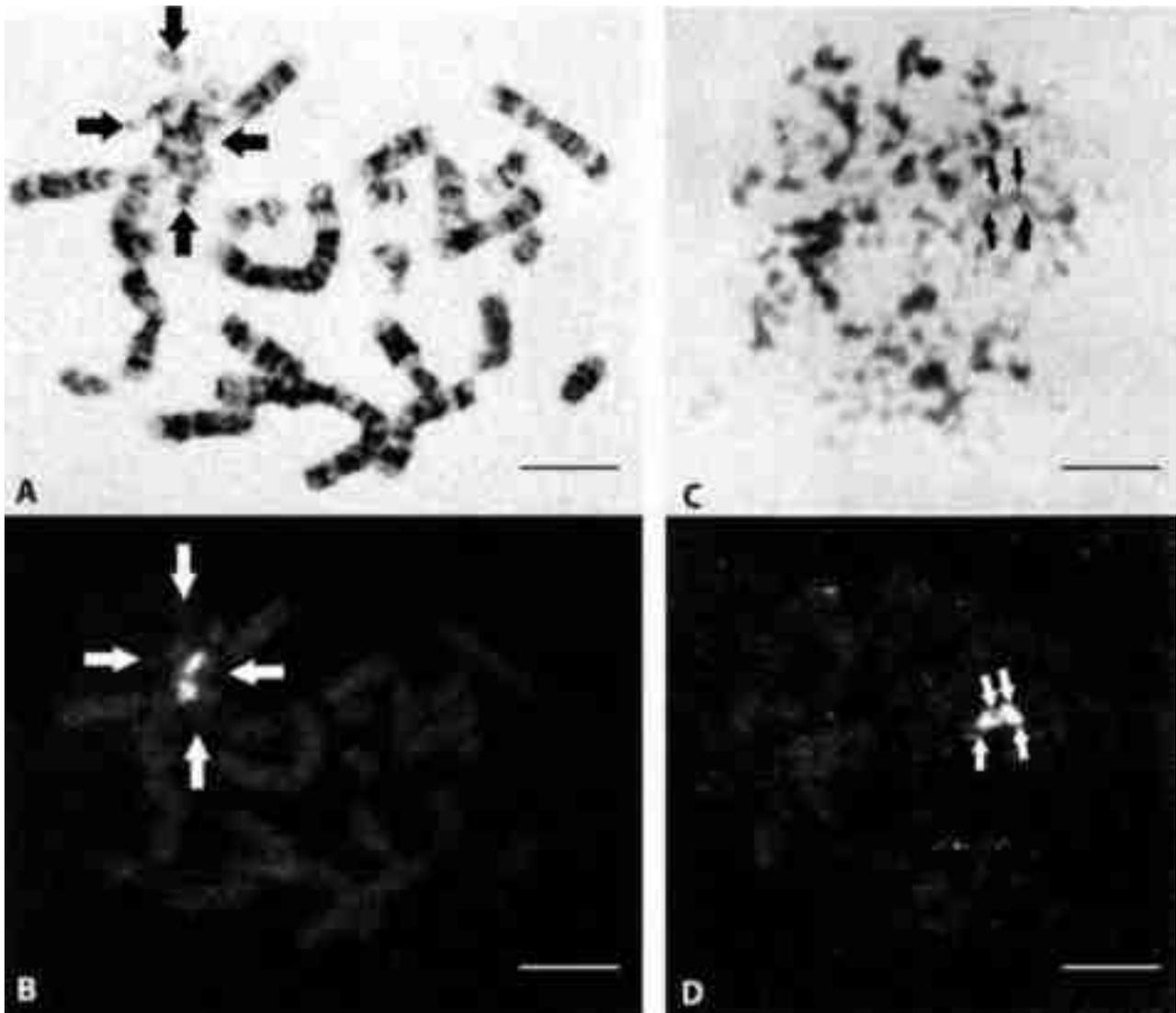
Figure 9. Immunocytochemical detection of microirradiated DNA in metaphase speads obtained after UV-microirradiation of cell nuclei during interphase. A,B) Giemsa stained metaphase spread (A) with partial chromosome shattering (PCS; arrows) obtained after microirradiation of the nucleus of a living M3-1 Chinese hamster and post-incubation for 6 hours in the presence of 2 mM caffeine. The same metaphase spread (B) after immunocytochemical detection of microirradiated DNA. Note its co-localization with the site of PCS (arrows) (reprinted from Cremer C., Cremer T. *et al.*, 1983 with permission). **C, D)** Giemsa stained mitotic M3-cell (C) with generalized chromosome shattering (GCS) was obtained following the same protocol shows immunolabelling of microirradiated DNA restricted to a small area (arrows) (reprinted from Cremer C., Cremer T. *et al.*, 1983 with permission). Bars correspond to 10 µm.

situ revealed autoradiographic label concentrated on a few chromosomes (average 4.3, range 1-7) (Figure 10). Chromosome segments labelled by UDS reflected the nuclear distribution of the labelled chromosomes at the nuclear site and time of microirradiation and provided unequivocal evidence for a territorial organization of interphase chromosomes. The finding that from two homologs only one was often heavily labeled, while the other remained unlabelled strengthened the evidence described above against Coming's hypothesis of a stable association of homologous chromosomes during interphase. Following microirradiation of a

single site at the nuclear edge, we observed metaphase chromosomes with UDS labelling at distal sites on both arms, while the centromeric region was free from label. This label pattern demonstrated that at the time of microirradiation the telomeric regions were located close to each other at the nuclear edge (Cremer T., Cremer C. *et al.,* 1982a).

UV-microbeam experiments in combination with the analysis of UDS provided an opportunity to analyze the stability/dynamics of higher order chromatin arrangements during interphase. Nuclei of fibroblastoid Chinese hamster cells were microirradiated at the two nuclear poles in G1, pulse-labeled
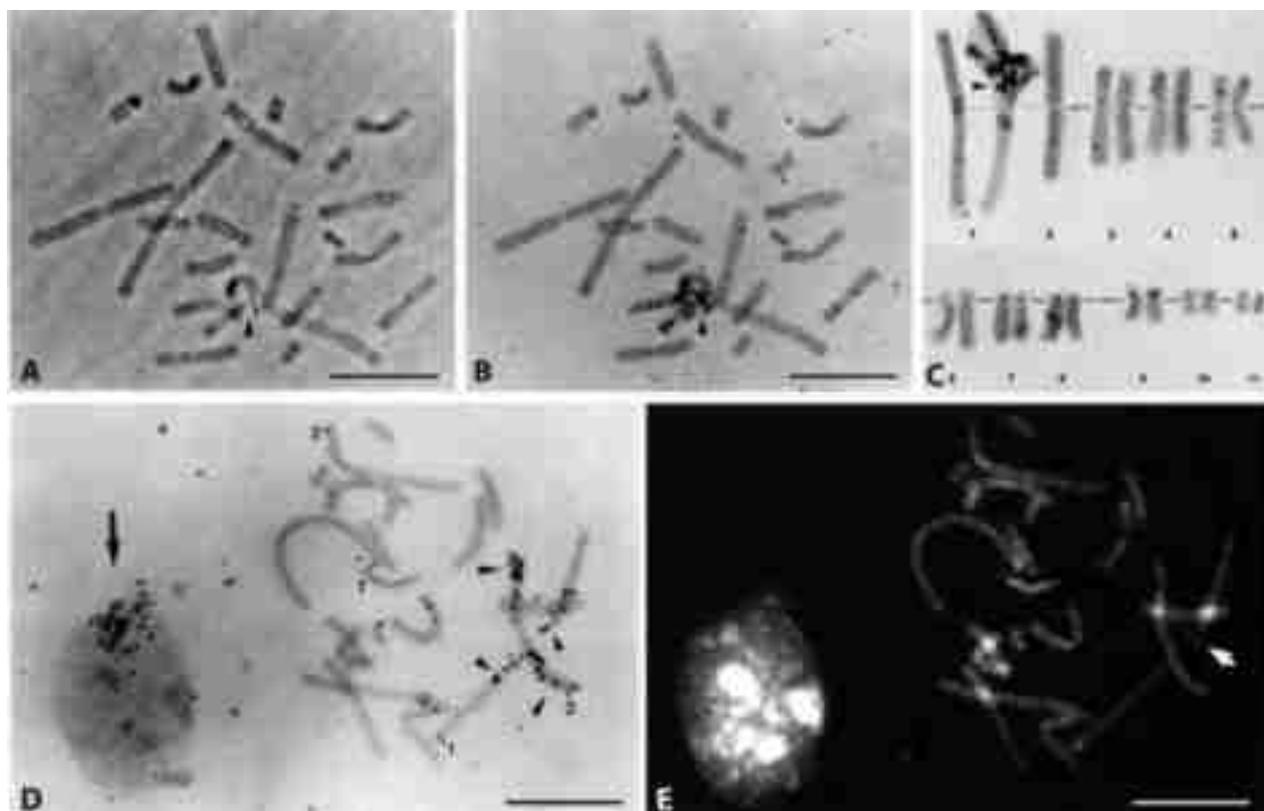
Figure 10. A-C) Phase contrast micrograph (A), autoradiograph (B) and corresponding karyogram (C) of a metaphase spread prepared *in situ* from a diploid, fibroblastoid Chinese hamster cell (reprinted from Zorn *et al.*, 1979 with permission). The cell was microirradiated at a single nuclear site during G1, pulse-labelled immediately thereafter with [³H] thymidine for 2 h and post-incubated for about 45 hours before it entered mitosis. Clusters of silver grains were noted over damaged segments of chromosomes 1 and 2 (arrows) indicating that unscheduled DNA synthesis (UDS) occurred in these segments after UV-microirradiation. We conclude that these labelled segments were selectively hit by the microbeam. **D, E)** Autoradiograph (D) and DAPI (4,6-diamidino-2-phenylindole dihydrochloride) fluorescence image (E) of an interphase nucleus and a metaphase spread about 40 hours following microirradiation of the two cells in G1 at the nuclear edge and pulse-labelling with [³H] thymidine for 2 h (reprinted from Cremer T., Cremer C. *et al.* 1982a with permission). The autoradiograph demonstrates UDS-labelling of the nucleus at the nuclear edge (arrow). Note the spatial association of two chromosomes 1 and 2 with intensively UDS-labelled segments, while their unlabelled homologues counterparts are located in a remote part of the metaphase spread. White arrow in (E) points to a gap or break in the labelled chromosome 1. Bars correspond to 10 μm.

with ³H-thymidine for 2 hours and processed for autoradiography either immediately (Figure 11A) or after an additional growth period 30 hrs (Figure 11B) (Cremer T., Cremer C. *et al.,* 1982a). Autoradiographs of these cells revealed the two microirradiation sites still at the nuclear poles. While this result argues against major post-irradiation movements of CTs, the more dispersed distribution of silver grains at the nuclear poles in (B) compared to (A) indicates local chromatin movements possibly as a consequence of CT replication during the 30 hour post-labelling period.

Evidence for CTs obtained by the analyisis of immunolabelling with antibodies specific against UV-microirradiated DNA

The UDS approach described could only be applied to cycling cells, which were microirradiated

outside S-phase. Antibodies specific for the detection of UV-irradiated DNA allowed us to demonstrate a territorial arrangement of Chinese hamster interphase chromosomes at all stages of interphase, including S-phase (Hens *et al.,* 1983). In further experiments, we microirradiated Chinese hamster cell nuclei at two sites, either remote from each other (mode A) or close to each other (mode B) and employed specific antibodies. After a postincubation period of 4 hours with caffein (2 mM) cells were fixed. At this time some cells had entered mitosis, while others were still in interphase. Microirradiated DNA was detected in interphase cells and mitotic cells by indirect immunofluorescence with primary antibodies showing a high affinity to UV-irradiated DNA (Figure 12A, B)(Cremer T., Baumann K. *et al.,* 1984a). These experiments supported the evidence described above for stable
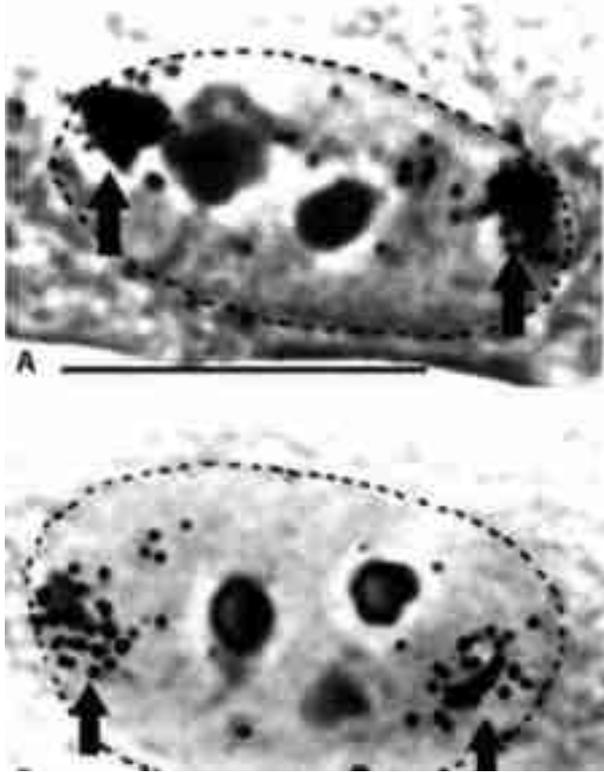
Figure 11. Evidence for stable positions of microirradiated chromatin during interphase. Autoradiographs of two Chinese hamster cell nuclei (reprinted from Cremer T., Cremer C.*et al.,* 1982a with permission) following laser-UV-microirradiation and pulse-labelling with [^{3}H] thymidine of cells in G1 at opposite nuclear poles. Silver grains (arrows) indicate that unscheduled DNA synthesis occurred in the two microirradiated nuclear regions both in a cell fixed immediately after the pulse (A) and in a cell fixed 30 hours later. Bars correspond to 10 μm.

CT arrangements during interphase (Figure 11) and for a remarkable clustering of microirradiated chromosomes in metaphase spreads (Figure 9, 10). The latter result can be interpreted either to show a special behaviour of damaged chromosomes or it may suggest that the neighbourhood of metaphase chromosomes generally reflects to some extent the neighbourhood of CTs during interphase. The relative distances between the two labelled sites were significantly larger ($p<$ 0.001) in 127 nuclei microirradiated at two sites far apart from each other compared to 104 nuclei microirradiated at two sites close to each other. Consistent with the interphase data, the relative distances between the two immunolabelled sites in 88 and 95 metaphase spreads metaphase spreads showed a signifcant difference ($p<$ 0.001) between spreads obtained after mode A and mode B microirradiation, respectively (Cremer T., Baumann H. *et al.,* 1984a). In addition to the UV-microirradiation experiments described

above, where we followed cells with microirradiated chromatin from interphase to metaphase, we also performed experiments, where we microirradiated a small part of the metaphase plate of living mitotic cells, allowed these cells to complete mitosis and observed the distribution of microirradiated chromatin in the two daughter nuclei several hours later (Figure 12C). The immunocytochemical localization of microirradiated DNA demonstrated that microirradiated chromosomes formed chromosome territories exhibiting mirror like patterns (Cremer T., Baumann H.*et al.,* 1984a).

In concluding this section we wish to emphasize a caveat, which must be taken into account with respect to any conclusions on chromatin dynamics based on the analysis of chromatin movements (or, in fact, the lack of major movements) observed after microirradiation of a given cell. We do not know whether microirradiation of nuclei had an inhibiting effect on subsequent chromatin movements. Therefore it can be argued that the relative stability of higher order chromatin arrangements during interphase, as well as the relationship between interphase and metaphase arrangements reflects the possibly aberrant behaviour of microirradiated chromatin and might not be representative for chromatin movements taking place in non-irradiated cells. In contrast, the analysis of metaphase chromosomes exhibiting chromosome segments labelled by UDS or antibodies specifc to UV-damage revealed unequivocal evidence, which CTs contributed to a given nuclear site at the time of microirradiation independent of any possible effects of microirradiation on interphase chromatin dynamics or artefacts of chromosome distribution during the preparation of metaphase spreads.

Generalized chromosome shattering following partial nuclear UV-irradiation: evidence for effects on the integrity of chromosome territories located outside the microirradiated nuclear area

Considering the compelling evidence in favour of a territorial organization of the cell nucleus provided by the UV-microbeam experiments described above, our observation of shattering of the entire mitotic chromosome complement (GCS) following microirradiaton of ca. 5% of the total nuclear area was a puzzle. These experiments suggested indirect effects of microirradiated chromosome territories on other territories located remote from the site of microirradiation. In order to suffer GCS cells with
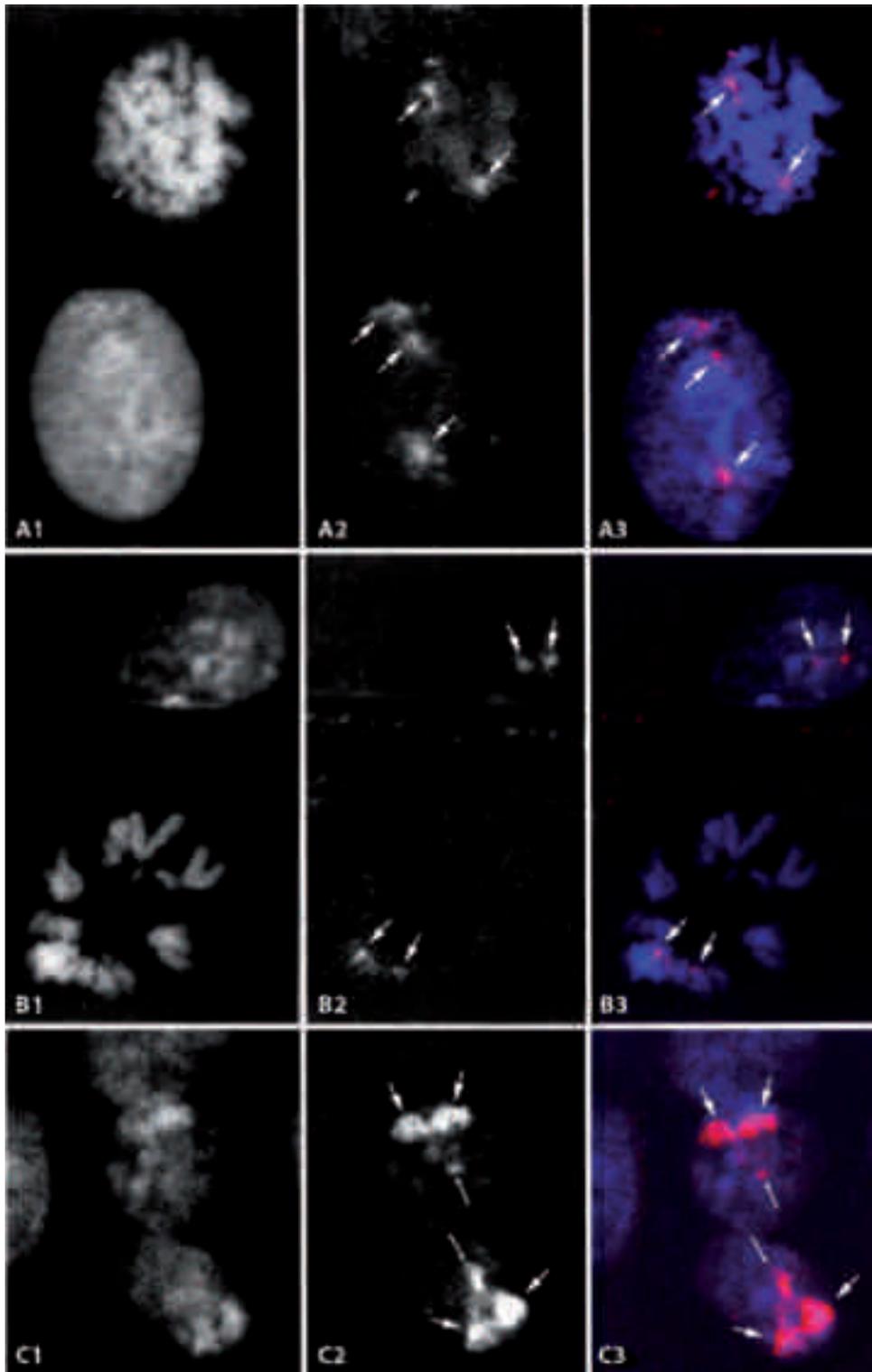
Figure 12. Evidence against large-scale movements of microirradiated chromatin during interphase. Nuclei of living Chinese hamster cells were microirradiated at two sites either far apart (panel A) or close to each other (panel B). Four hours later cells, which had entered mitosis side by side with cells still in interphase were fixed and stained with DAPI (A1, B1) together with the immunocytochemical detection of microirradiated DNA (A2, B2; arrows)(reprinted from Cremer T., Baumann H. *et al.*, 1984a). Panels A and B show one cell during mitosis and a nearby nucleus from an interphase cell. False coloured image overlays (A3, B3) of A1/A2 and B1/B2 demonstrate a correlation between the distances between the two microirradiated sites in interphase nuclei and in mitotic cells (blue: DAPI, red: microirradiated DNA). Panel C: Laser-UV-microirradiated metaphase chromosomes form chromosome territories in daughter nuclei. Panel C: shows a pair of daughter nuclei fixed 3 h 45 min after microirradiation of a small part of the metaphase plate of a living M3-1 Chinese hamster cell. Nuclei were stained with DAPI (C1) following the immunocytochemical localization of microirradiated DNA (C2; arrows). False coloured image overlays (C3) demonstrate a mirror like distribution of microirradiated chromatin consistent with the formation of chromosome territories (reprinted from Cremer T., Baumann H. *et al.*, 1984a, with permission).
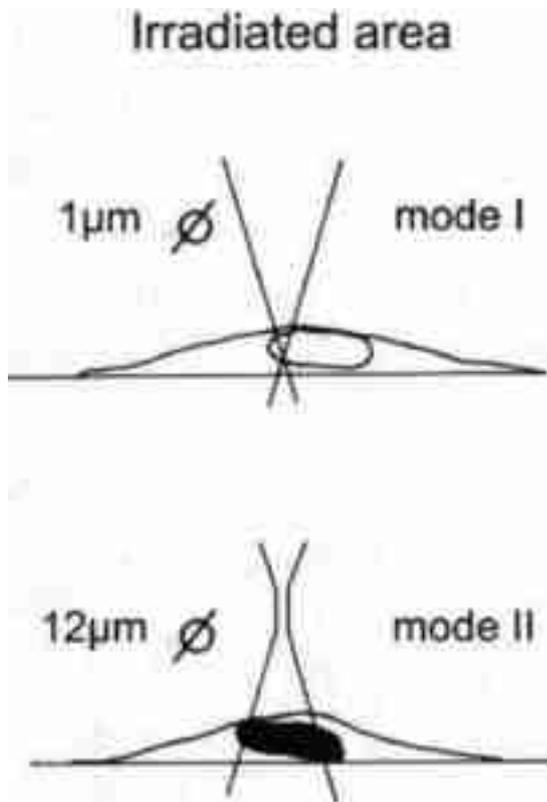
Figure 13. Mode I and mode II microirradiation of cell nuclei Laser-uv-microirradiation of the nucleus was performed either by focussing the beam into the cell nucleus (mode I) or by de-focussing the beam to an extent that a large part of the nucleus was microirradiated. While the UV-incident energy was the same the energy density in the exposed part of the nucleus differed by a factor of ~20. (Cremer C., Cremer T., Jabbur G.)

microirradiated nuclei needed to transverse S-phase after the microirradiation event. We first speculated that cells with GCS resulted from a premature condensation of S-phase chromosomes. DNA measurements of cells with GCS, however, yielded a normal G2/M DNA content (Cremer C. and Gray, 1982) and argued that cells with GCS had completed DNA-replication, at least to the most part. Furthermore, videorecording of living cells indicated failed attempts of mitotic chromosome movements in cells with GCS (unpublished data). The cells, however, were not stuck in mitosis, but able to form a number of micronuclei with sizes suggesting that they might each contain several chromosomes (Cremer C, Cremer T. et al., 1980b). The unexpected lack of a large fraction of micronuclei typical for chromosomal fragments emphasized that shattered chromosomes were not the consequence of a large number of true chromosome or chromatid breaks. These results together with the notion that caffein reduces the G2 period prompted

the hypothesis that chromosome shattering in microirradiated cells represents a failure of normal chromatin condensation of the entire chromosome complement, when such cells enter prophase. Next, we performed UV-microbeam experiments, where a given incident energy was either concentrated to a small part of nucleus (mode I) or distributed over approximately the whole nuclear area (mode II) (Figure 13). Cells were posttreated with 1 or 2 mM caffein until mitosis. Compared with mode II the local UV-fluence in mode I experiments was two orders of magnitude higher. Cases of PCS were only observed after mode I microirradiation, while GCS was obtained after both mode I and mode II microirradiatiion. The size of the fraction of mitotic cells with GCS increased with the total incident energy and was independent of the distribution of repair sites (Cremer C., Cremer T. et al., 1981b).

The distribution of DNA photolesions at a small nuclear site (mode I) or throughout the entire nuclear space (mode II) (Figure 13) resulted in roughly the same quantitative response of unscheduled DNA synthesis. Autoradiographic experiments with ^{3}H-thymidine pulses showed within the investigated range of energy densities in mode I and II experiments (2.7–1000 J/m^2), that the total amount of unscheduled DNA synthesis was correlated with the total incident energy but not with its distribution (Cremer C, Cremer T. et al., 1981b).

In an attempt to explain the unexpected phenomena described above, we developed a factor depletion model (Cremer C. and Cremer T., 1986a; Cremer C, Cremer T. et al., 1980b) (Figure 14). We argued that different sets of repair factor(s) for excision repair and postreplication repair, respectively, can freely move in the nuclear sap. The available pools of repair factors are variable from cell to cell. Depending on factor pool sizes available for excision or post-replication repair, each cell can deal with a certain number of DNA-photolesions. With regard to PCS or GCS the generation of postreplication repair (PRR) sites during S-phase in microirradiated chromatin is essential. PRR sites compete for the binding of essential factors with other sites X of a still unidentified molecular nature. These X-sites are generated during S- and possibly also during G2-phase throughout the entire genome. Note that the formation of PRR- and X-factor-binding sites requires that cells with microirradiated nuclei pass through S-phase. We argue that in microirradiated cells grown in the absence of
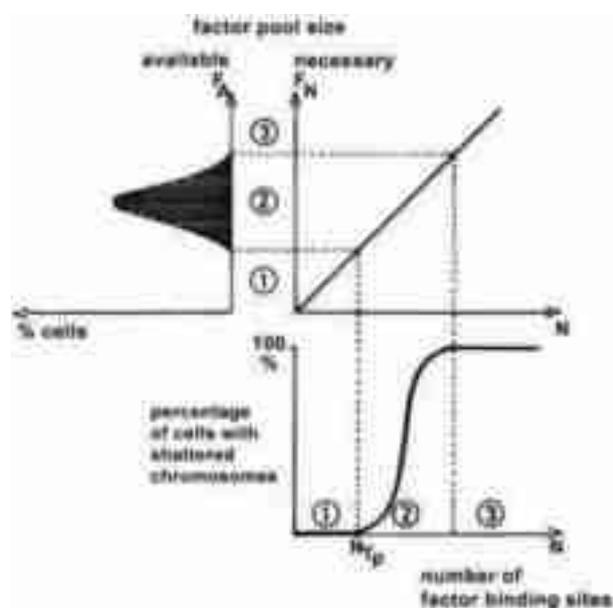
Figure 14. Factor depletion model of generalized chromosome shattering (GCS). The factor depletion model was developed as an attempt to explain that GCS was observed in Chinese hamster cells both after mode I and mode II laser-UV-microirradiation (Cremer C. and Cremer T., 1986a; Cremer *et al.,* 1980b) (see Figure 13; adapted from Zorn, 1978). According to this model the nucleus contains a critical number of factors F freely moving in the nuclear sap. F is essentially required not only at sites of post-replication repair (PRR) of DNA-photolesions, but also at other sites X throughout the entire genome. Both PRR-sites and X-sites are generated during S-phase throughout the entire genome. They compete with each other for the binding of the unknown factor(s). Inhibition of PRR with 1-2 mM caffein blocks or at least delays the release of F from PRR-sites, while the generation of factor F binding X-sites continues. When the F-pool available in the nucleus of a given cell becomes exhausted, GCS is triggered due to an undersupply of newly generated X-sites. We assume that the size of the F-pool varies in different cells and for simplicity that the percentages of cells with a given pool size follow a normal distribution. In unirradiated cells and in cells exposed to a low UV-incident dose (range 1) the F-pool is sufficiently large in all cells to avoid GCS. With increasing UV-incident dose (range 2) the percentage of cells, in which the F-pool becomes exhausted increases from 0% to high levels and the percentage of mitotic cells with GCS increases accordingly. Finally, at even higher UV-incident doses (range 3) the F-pool becomes exhausted in all UV-irradiated cells, which pass through S-phase after UV-exposure. Note that according to this model induction of GCS critically depends on the number of PRR- and X-sites that accumulate in a given nucleus but not on their distribution. Accordingly, mode I and mode II laser-UV-microirradiation with a given incident UV-dose will have the same effect. The molecular nature and function of F remains unknown to date.

caffein the F-pool size is sufficient to serve all factor binding sites, whether they are formed in microirradiated or non-irradiated chromatin of the nucleus. Post-treatment with caffein at a concentration range (1-2 mM) has no detectable effect on the chromosomal integrity of non-irradiated control cells, but leads to a delayed termination of PRR-sites and consequently to a delayed release of factors transiently bound at these sites. This effect of caffein combined with the additional generation of

factor binding X-sites in non-irradiated chromatin may result in an undersupply of the hypothetical factor molecules not only at PRR-sites but also at X-sites generated in all interphase chromosomes. According to our model this undersupply finally triggers GCS. The model predicts that cell cycle stage at the time of UV-microirradiation, the caffein concentration and the number of DNA photolesions, but not their distribution in the cell nucleus are important parameters for the induction of GCS (Cremer and Cremer, 1986a; Cremer T., Cremer C. *et al.,* 1980b). We argue that cells with PCS occur under conditions, where the size of the factor pool is just sufficient to supply all X-sites generated in non-irradiated CTs with sufficient amounts of F, while a critical accumulation of both PRR- and X-sites occurs in UV-microirradiated CTs. Accordingly, unirradiated interphase chromsomes undergo a normal condensation process, while shattering of a few UV-microirradated chromosomes leads to PCS. The molecular nature and function of the hypothetical factor(s) remains unknown to date. The observation that cells with GCS apparently enter mitosis and show normal G2/M DNA contents suggests that F participates in post-replicative chromatin modifications necessary for the normal condensation of mitotic chromosomes or may be directly required for normal prophase chromosome condensation.

The observation of GCS in microirradiated nuclei prompted experiments to answer the question, whether UV-microirradiation of cell nuclei would induce increased numbers of sister chromatid exchanges in the entire chromosome complement or restricted to microirradiated chromosomes. Our respective experiments support the latter assumption (Figure 15) (Raith *et al.,* 1984).

Evidence for a territorial organization of chromosome territories by *in situ* hybridization experiments

Despite their usefulness to study the higher order organization of chromatin, UV-microbeam experiments had two major disadvantages: they requested equipment lacking in most other laboratories and they could only be applied for studies of *in vitro* cultivated, cycling cells. A method was needed to visualize any chromosome and chromosomal subregion of interest directly in the cell nucleus. For humans and other mammalian species possibilities to observe the location of specific chromosomes or
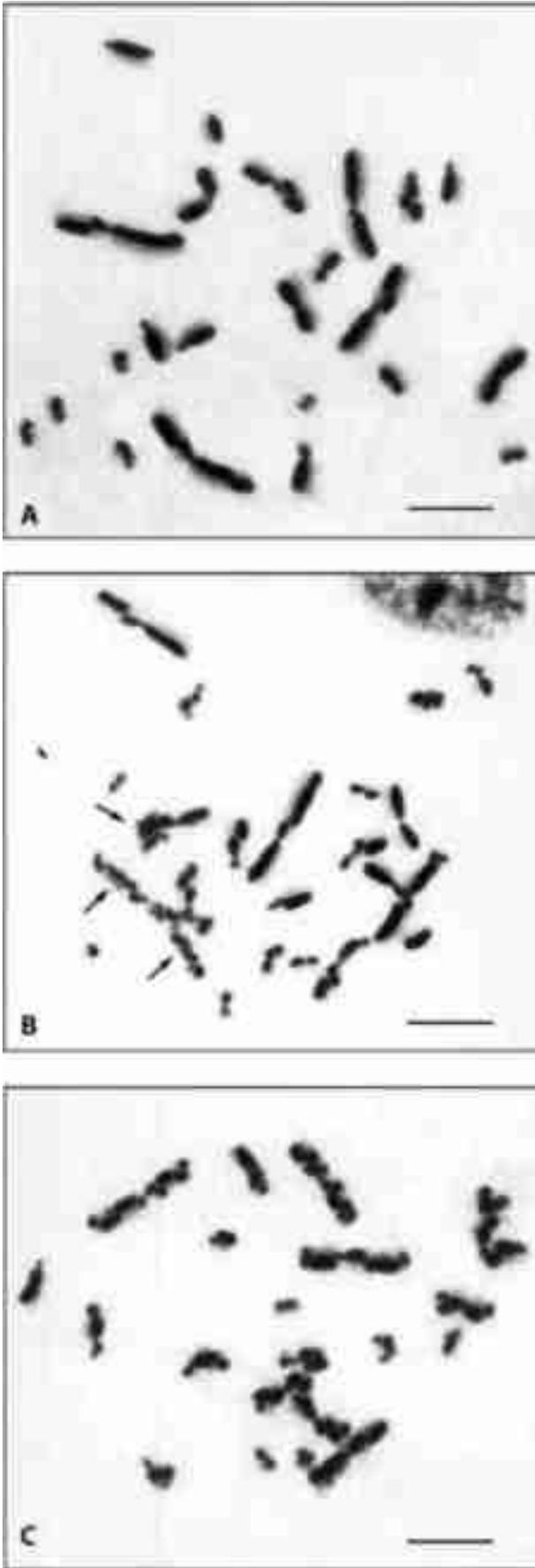
chromosomal segments were still restricted in the early 1980s to the Barr body (representing the inactive X chromosome) (Barr and Bertram, 1949) and a few heterochromatic segments, such as the heterochromatic block on the long arm of the human Y chromosome and on HSA1q12 and 9q12 (Hoehn and Martin, 1973; Spaeter, 1975). In 1975 and 1976 Michael Schmid and co-workers published seminal papers on the nuclear architecture of spermatid nuclei from amphibian (Urodeles) and bird species (Gallus domesticus) (Dressler and Schmid, 1976; Schmid and Krone, 1975; Schmid and Krone, 1976). They demonstrated a correlation of spermatid differentiation with the relocation of individual chromosomes marked by a specific heterochromatic block. This block was first observed at one site of the nucleus together with other blocks of heterochromatin (suggesting a Rabl orientation), but then moved to the other site of the nucleus, where the acrosome of the sperm was subsequently formed. While the biological significance of this phenomenon has remained unclear to date, this early observation provided a striking example for the directed movement of an individual chromosome in the cell nucleus and showed that chromosome positions in the cell nucleus cannot be generally explained as a consequence of their anaphase/telophase movements. The direct visualization of chromosomes and chromosomal segments in the nucleus and the development of isotopic *in situ* hybridization approaches (Gall and Pardue, 1969) and the advent of non-isotopic ISH methods in the early 1980s (Bauman *et al.,* 1980; Langer *et al.,* 1981), for review see (van der Ploeg, 2000) provided the methodological basis to visualize in principle any specific chromosomal subregion of interest (Figure 16) and even entire, individual chromosomes (Figure 17) not only in mitotic cells but also in nuclei of cycling or postmitotic cells (Burns *et al.,* 1985; Cremer T., Landegent J. *et al.,*

Figure 15. (Left) Laser-UV-microirradiation induces sister chromatid exchanges in microirradiated chromosome territories. Distribution of sister chromatid exchanges (SCEs) in Chinese hamster metaphase spreads (line M3-1) (Raith *et al.*, 1984). Cells were grown for 11 hours in medium supplemented with 10 µg/mL bromodeoxyuridine (BrdU) before microirradiation was performed. Metaphase spreads were prepared *in situ* after further incubation of the cells in BrdU-free medium for 13 hours. A) Control metaphase spread. B) Metaphase spread following microirradiation of about 30% of the nuclear area at one nuclear pole during the preceding interphase. Arrows indicate chromosomes with a strongly increased density of SCEs compared to controls. C) Metaphase spread following microirradiation of approximately the whole nuclear area during the preceding interphase. Most chromosomes show multiple SCEs. Bars correspond to 5 µm.
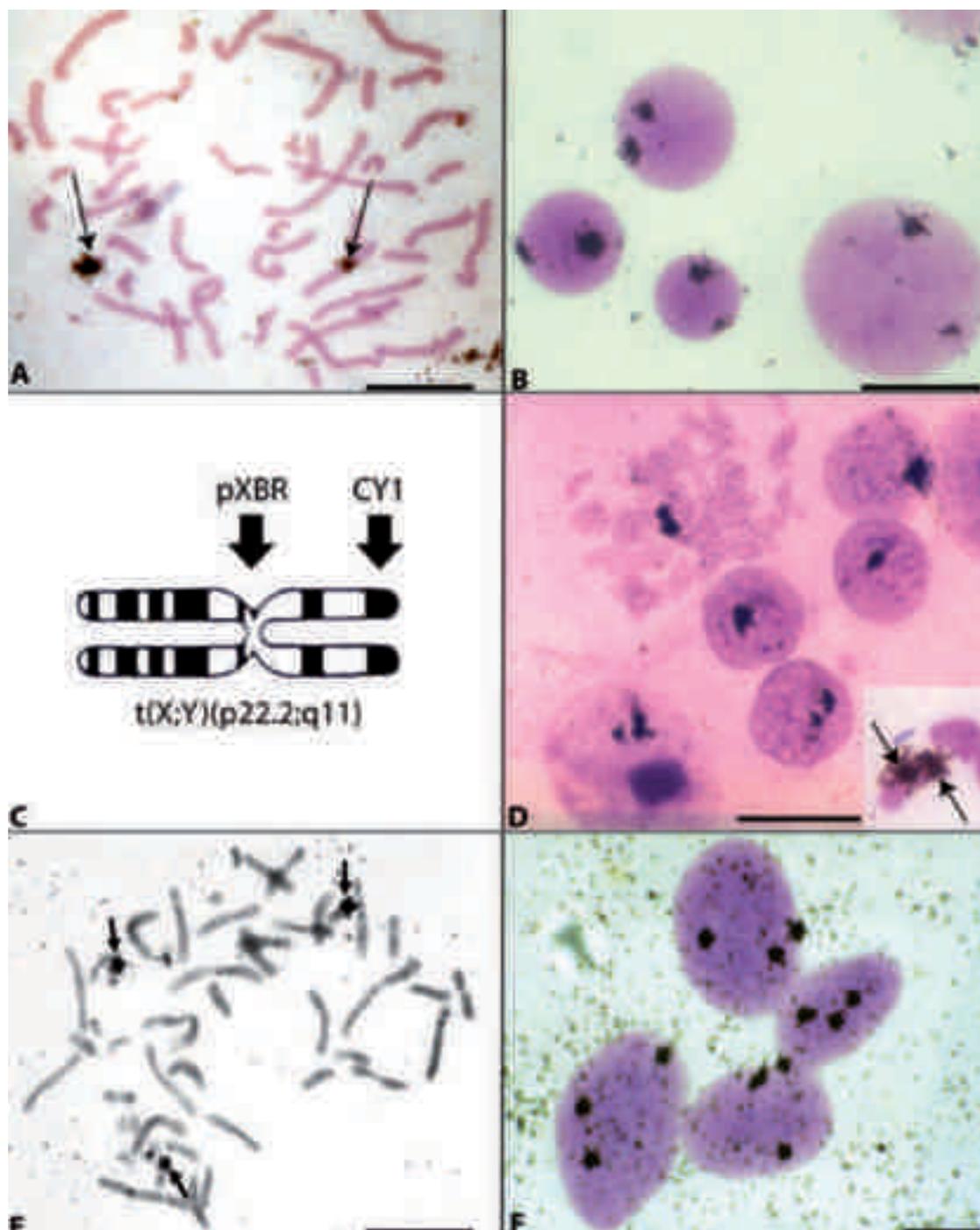
Figure 16. Radioactive *in situ* hybridization with chromosome specific DNA probes allows the specific detection of the hybridization sites in both metaphase spreads and interphase nuclei. A, B) Autoradiographs of a Giemsa stained human metaphase spread (46,XY) (A) and of four PHA stimulated, human lymphocyte nuclei (B) following radioactive *in situ* hybridization with the probe pXBR specific for X-pericentromeric, repetitive sequences and probe CY1, which delineates repetitive sequences on Yq12 (arrows) (Rappold *et al.,* 1984b). Since the signals detected in metaphase spreads on the Y chromosome were clearly stronger than the signals on the X chromosome, we suggest that the larger of the two signals detected on interphase nuclei also delineates the Y. **C)** Scheme of a human translocation chromosome (Xqter→Xp22.2:Yq11→Yqter) contained in a hybrid Chinese hamster x human cell line with the chromosomal positions of probes pXBR and CY1 (Rappold *et al.,* 1984b). **D)** Autoradiograph of a Giemsa stained metaphase spread and several interphase nuclei from the hybrid cell line described above following radioactive *in situ* hybridization with probes pXBR and CY1. Compatible with a territorial organization of the translocation chromosome distances between the two labeled sites observed are similar in interphase nuclei and metaphase spreads. The inset shows an enlarged t(X;Y) metaphase chromosome with arrows pointing to the two hybridization signals (Rappold *et al.,* 1984b). **E, F)** Autoradiographs of a metaphase spread (E) and of four interphase nuclei (F) from cultured human amniotic fluid cells with a trisomy 18 following radioactive *in situ* hybridization with a probe (L1.84) specific for the detection of repetitive, pericentromeric sequences of HSA 18 (Cremer T., Landegent J. *et al.,* 1986b). Arrows in A indicate clusters of silver grains over three chromosomes 18. Three signals are also seen on each of the four interphase nuclei. Bars correspond to 10 µm.
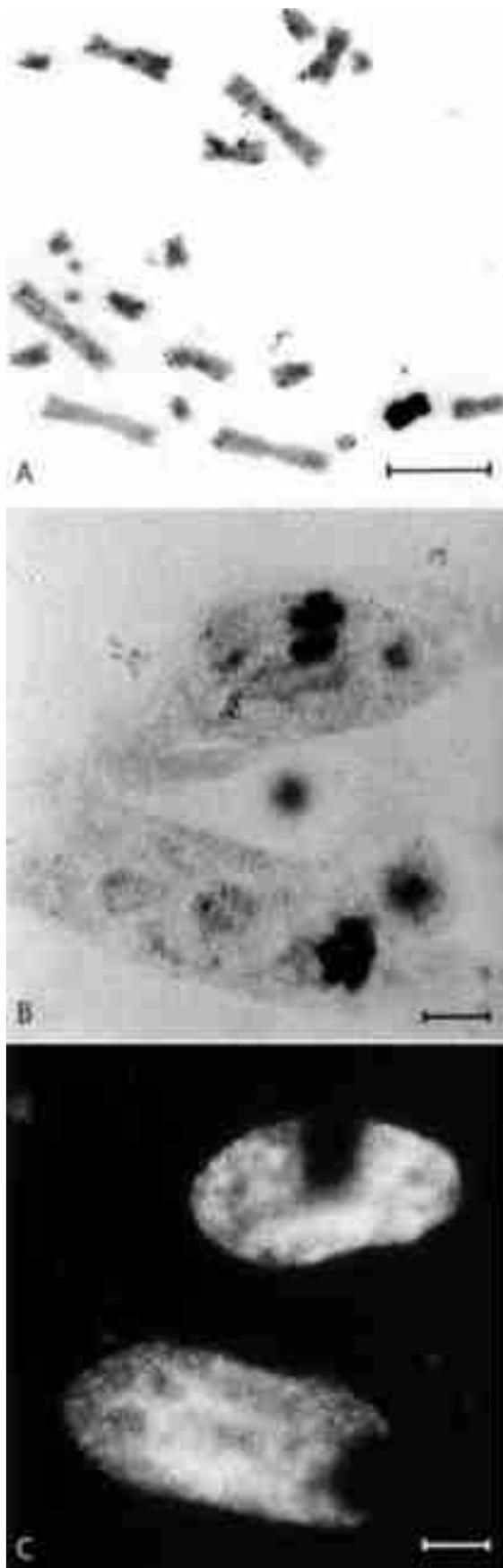
1986b; Manuelidis, 1985; Pinkel *et al.,* 1986; Rappold *et al.,* 1984a; Schardin *et al.,* 1985). In 1986 this approach was termed "interphase cytogenetics" (Cremer T., Landegent J. *et al.,* 1986b). Today it is mostly referred to as interphase FISH. In retrospect the term "nuclear cytogenetics" seems more appropriate to include its feasibility to study chromosomal aberrations present in nuclei of post-mitotic and terminally differentiated cells as well. Notably, the discovery that chromosomes and chromosomal subregions show a territorial organization in the cell nucleus was essential for the realization of interphase cytogenetics as a widely applicable diagnostic concept. If chromosomes would decondense in the cell nucleus to an extent that chromatin fibres intermingle like spaghetti in the soup, one would expect smeared signals and it would not be possible to count aberrant numbers of chromosomes and chromosomal subregions. Figure 18 exemplifies the diagnostic possibilities of interphase cytogenetics envisaged in 1985. These included 1. the use chromosome specific alphoid DNA probes to demonstrate the presence of 1, 2 or 3 segments of centromeric heterochromatin from a specfic chromosome of interest (Figure 18A), as demonstrated for chromosome 18 (Figure 16E,F) (Cremer T., Landegent J. *et al.,* 1986b), 2. the differential colouring of entire individual chromosomes of interest as a means to analyze the copy number of these chromosomes, as well chromosomal rearrangements (Figure18B,C), 3. the detection of specific translocation breakpoints directly in the cell nucleus (Figure 18D,E) (Cremer *et al.,* 1986b): "By using nested sets of chromosome specific DNA probes and a bi-coloured detection of two chromosomes or chromosomal sub-regions (Hopman *et al.,* 1986), one chromosome or sub-region might be visualized with green the other chromosome or sub-region with red fluorescence.

Figure 17. (Left) Non-radioactive *in situ* hybridization with human genomic DNA reveals the human X chromosome in a Chinese hamster x human hybrid cell line carrying a single, transcriptionally active human X chromosome. A) Metaphase spread reveals a single, intensely stained human X chromosome following non-radioactive *in situ* hybridization with biotinylated human genomic DNA as a probe. The hybridized probe was detected by an alkaline phosphatase generated colour precipitate (for details see Schardin *et al.*, 1985). Chinese hamster chromosomes are slightly coloured with Giemsa stain. B,C) Transmission light microscopy of two interphase nuclei shows the distinctly coloured human X-territory (B). For epifluorescence microscopy (C) nuclei were counterstained with DAPI. The dark area in each nucleus represents the human X-territory, where the precipitate generated by alkaline phosphatase blocks the DAPI fluorescence. Bars correspond to 10 μm.
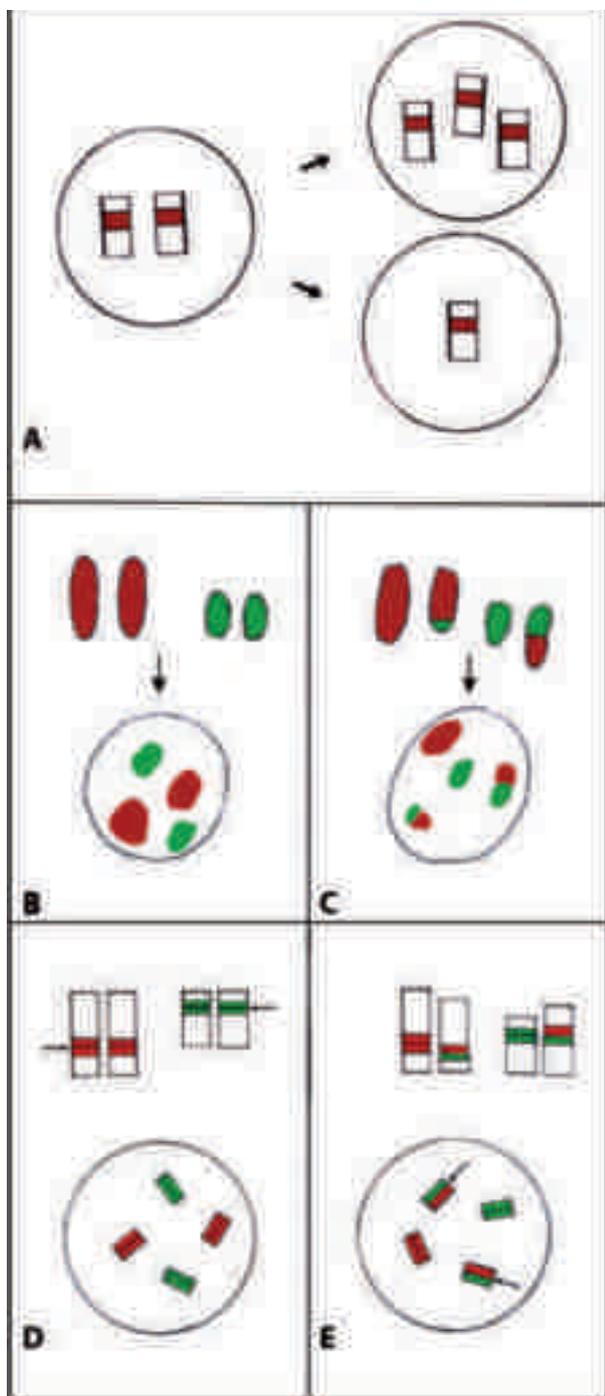
Figure 18. Diagnosis of numerical and structural chromosome aberrations in the cell nucleus: the concept of interphase cytogenetics (Cremer *et al.*, 1986b) (schemes from T. Cremer 1985) A) Colouring of a specific chromosomal segment, e.g., constitutive heterochromatin of a given chromosome allows the identification of numerical aberrations, such as mono-somies or trisomies. B,C) The differential colouring of two individual chromosomes allows the identification not only of numerical aberrations of these chromosomes, but also of structural rearrangements. D,E) In cases, where the two breakpoints of a specific reciprocal translocation are known, the presence of this translocation can be analyzed in the cell nucleus using two colour FISH with DNA probes, which over-lap the two breakpoints. The normal situation is shown in D, the translocation event in E.

Normal interphase nuclei should represent two green and two red fluorescent spots with variable distances. Consider a specific translocation between these chromosomes. We predict that interphase nuclei containing the specific translocation should bear *three* green and *three* red fluorescent spots. The distribution of each set of spots is expected to be variable depending on the presumably variable positions of the chromosomes involved. However, two green and two red spots should be detected side by side, since they mark the positions of the two translocation chromosomes. The third green and red spot, respectively, would indicate the position of each normal homologue''. Since then fluorescence *in situ* hybridization (FISH) has been widely used by us and others to discover numerical and structur-al aberrations at any stage of the cell cycle and also in nuclei of post-mitotic cells, including terminally differentiated cells in tissue sections (for reviews see (Cremer and Cremer, 2001a; Cremer *et al.*, 2006a; Cremer *et al.*, 2000; Cremer *et al.*, 2004; Cremer *et al.*, 1993). In the 1980s the broad application of interphase cytogenetics was still hampered by the lack of DNA probes of different complexities able to visualize specific DNA from whole chromosomes to arms to band to genes of interest directly in the nucleus.

Chromosome sorting and chromosome painting: development of tools to visualize individual chromosomes in the cell nucleus

The development of chromosome sorting in the 1970s (Gray *et al.*, 1975; Lebo *et al.*, 1979) and the availability of phage libraries from sorted human chromosomes in the mid 1980s suggested a way to use the complex sequence content of such libraries to visualize individual chromosomes. The first phage clone library from the human X chro-mosome was reported in 1981 (Davies *et al.*, 1981) One of us (C. Cremer) had contributed at the Lawrence Livermore National Laboratory during the early 1980s to the further development of chro-mosome sorting and succeeded to sort several hun-dred thousand copies of the human Y chromosome. This number was needed at that time to establish a representative phage clone library for an individual chromosome (Cremer C., Rappold G. *et al.*, 1984b). Using radioactive *in situ* hybridization we could confirm that several individual clones of our human Y clone library hybridized as expected to the human Y (Cremer C., Rappold G. *et al.*, 1984b; Rappold *et*

al., 1984a). In the meantime a number of human autosome libraries had also become available (Krumlauf *et al.,* 1982). Today a few hundred chromosomes suffice to establish a clone library with rather full sequence coverage. It has also become possible to generate libraries from microdissected chromosomes or chromosome parts with high efficiency (Guan *et al.,* 1996; Meltzer *et al.,* 1992) and even to generate sequence pools from a single microdissected chromosome with sufficient complexity to paint the respective chromosome (Guan *et al.,* 1993; Schermelleh *et al.,* 1999).

Although the use of the complex library DNA from sorted chromosomes as a probe for the visualization of entire chromosomes was an obvious possibility in the early 1980s, it was also obvious that chromosome libraries even from very pure sorts contained a large fraction of repetitive sequences, which were not restricted to the target chromosome, but distributed over the whole genome. As a consequence, such a library DNA probe was expected to hybridize not only to the target chromosome, but to many other chromosomes as well. A FISH workshop organized 1984 in Paris by Giorgio Bernardi, Laura Manuelidis and David C. Ward provided an opportunity to discuss this problem and the intriguing horizon of possibilities, which could result from its solution for the future of the then emerging new field of molecular cytogenetics. This meeting resulted in the invitation of T. Cremer by Laura Manuelidis and David Ward to pursue a common project as a Visiting Professor at Yale University. This team work started in spring of 1986 with the support of a Heisenberg stipend granted to T.C. from the Deutsche Forschungsgemeinschaft (DFG). In the application for this prestigious stipend (Cremer T., 1985) two procedures were proposed, which should lead to the selective visualization of chromosomes both in mitotic cells and in cell nuclei. The first approach was based on affinity chromatography protocols routinely established in the Ward laboratory: "The removal of non-chromosome specific sequences [from a DNA clone library established after sorting of a given chromosome type, e.g. the X] will be carried out with the help of affinity chromatography. For this purpose DNA libraries from other chromosomes will be used (alternatively Cot-fractions with repetitive human genomic DNA are also taken into consideration). Phage- or plasmid clones from these libraries will be nick-translated with nucleotides conjugated with

biotin. In a next step biotinylated DNA will be denatured and a great excess of it will be hybridized [in solution] to the denatured, unlabelled DNA of the X chromosome library. After hybridization [is achieved] as complete as possible, affinity chromatography using avidin-sepharose or anti-biotin IgG-sepharose columns is performed to remove quantitatively all biotin-labelled hybrid DNA molecules and all remaining single stranded biotin-labeled sequences (these columns are available in David Ward's laboratory). The remaining fraction should be strongly enriched with regard to X-chromosome specific sequences. Non-chromosome specific sequences of the X-chromosome DNA library, which by chance hybridize with each other, will, of course, escape removal by affinity chromatography. Such event, however, should be rare in case that the biotinylated sequences are present during hybridization in large excess (e.g. 2000:1). If necessary, the procedure can be repeated with the [enriched] fraction [of X-specific sequences] until the specificity of the final fraction meets the demands. This fraction can then be cloned and used for the specific visualization of the [human] X in the same way as the human X has already been visualized in hybrid cells."

A second, apparently simpler strategy was also outlined in the application for the Heisenberg stipend (Cremer T., 1985): "Here, the DNA sequences from a library of sorted X-chromosomes will be nick-translated with biotin labelled nucleotides within their vectors and directly used for *in situ* hybridization. In order to avoid the hybridization of biotin-labelled, but not chromosome specific sequences to the entire chromosome complement, a suitable carrier DNA will be added in excess. This carrier DNA comprises non-biotinylated inserts, which can be selected from the DNA libraries of other chromosomes. ... Modifications of this strategy are conceivable in many ways. As a carrier DNA one may simply try, for example, Cot-fractions of highly repetitive human DNA. ... The feasibility of the strategy in principle seems to be beyond question. ... Since it is impossible to ponder the possible advantages and disadvantages of all different modifications on the basis of theoretical considerations, is situ hybridization experiments must finally decide, whether the specificity of a DNA sequence fraction and of the applied carrier DNA, respectively, suffices to visualize the chromosome in question."

Our team at the Yale University was soon greatly strengthened in 1986 by Peter Lichter, who became fascinated by our goal and started postdoctoral research in David Ward's laboratory. Attempts to remove repetitive sequences with genome wide distribution from a HSA 21 library using various modifications of affinity chromatography protocols failed. The results of FISH with the resulting probes were consistently disappointing: in addition to the targeted chromosome 21 the whole chromosome complement flashed up. While we could prove an enrichment of sequences specific for the HSA 21 target chromosome, this progress was foiled by the fact that our affinity columns retained only about 99% of the excess of repetitive, biotinylated sequences, but not 100%, as we had hoped for. Using an excess in the order 100:1 of biotinylated sequences from phage libraries of other non-targeted chromosomes compared to the amount of non-biotinylated sequences from the HSA 21 target library, a 1% flow through of repetitive, biotinylated sequences was by non means negligible. It always added to the enrichment of HSA 21 specific sequences and was sufficient to spoil our hopes.

Independent from our own efforts, the group of Joe Gray and David Pinkel had also pursued the goal of a specific visualization of chromosomes using the second approach described above. This approach made direct use of the DNA prepared from the phage library established from the DNA of a given sorted chromosomes as a FISH probe and turned out to be extremely rewarding (Pinkel *et al.,* 1988). As mentioned above the unwanted hybridization of biotinylated or otherwise labelled repetitive sequences present in all phage libraries from sorted human chromosomes to non-targeted chromosomes was suppressed by an excess of unlabelled Cot-1 DNA. To emphasize this fact, we called the protocol developed in parallel in the Manuelidis and Ward laboratories "chromosomal *in situ* suppression (CISS) hybridization" (Cremer T., Lichter P. *et al.,* 1988b; Lichter *et al.,* 1988a), but the more elegant and artful term "chromosome painting" invented by (Pinkel *et al.,* 1988) sticked and is now in general use. Figure 19A-D presents examples of painted human chromosomes 7 together with chromosome 7 centromeres in nuclei of normal, diploid cell nuclei (Figure 19A,B) (Lichter *et al.,* 1988a) and tumor cell nuclei, respectively (Figure 19C,D) (Cremer T., Lichter P. *et al.,* 1986b). Figure 19E shows two painted X-chromo-

somes, the metaphase spread of a female individual together with an interphase nucleus showing the two X-territories. Arrows point to the localization of the dystrophin gene. Figure 19F provides an example of a female, who is a carrier for Duchenne muscular dystrophy. This female was the mother of a boy with this disease. Molecular studies had revealed that the boy had a deletion within the dystrophin gene. FISH with a cosmid probe specific for the deleted region revealed a signal only on one of the two X-chromosomes (Ried *et al.,* 1990).

For routine applications the use of commercial sources of Cot-1 DNA was expensive and the efficiency of different batches varied. For this reason, we felt it still useful to generate chromosome painting probes, which can be applied without Cot-1 DNA. A solution of the "flow through" problem described above became possible, when we combined affinity chromatography with the specific amplification of enriched sequences from targeted chromosomes (Bolzer *et al.,* 1999).

Breaking the Abbe limit of conventional light microscopy: early concepts and developments

For cell biologists and histochemists ideas to improve the resolution of light microscopy are certainly of great interest, if they have a smack of feasibility. The resolution limit, however, defined by the famous formula of Ernst Abbe seemed to defy any dreams of this kind (Abbe, 1873). This Abbe limit postulates that the smallest distance resolvable in the object plane between any two point-like objects cannot be much smaller than about half the wavelength of transmitted light used for the illumination of the objects, i.e. a few hundred nm using visible light. The same conclusion holds for fluorescent objects (Rayleigh theory of resolution). For biologists and even for most physicists in the early 1970s the Abbe limit seemed to reflect fundamental principles of the physics of light, which restrict the possible structural resolution of the light microscope, i.e. its capability to distinguish neighbouring elements, which are located at a distance smaller than roughly half the wavelength of the light. Intellectual efforts to find technical tricks to overcome this limit even had the smack of disregarding principle laws of nature similar to proposals of building a machine to solve the energy problems of mankind by the *de novo* generating of energy. While the rise of electron microscopy in the 1940s and 1950s improved resolution dramatically, this
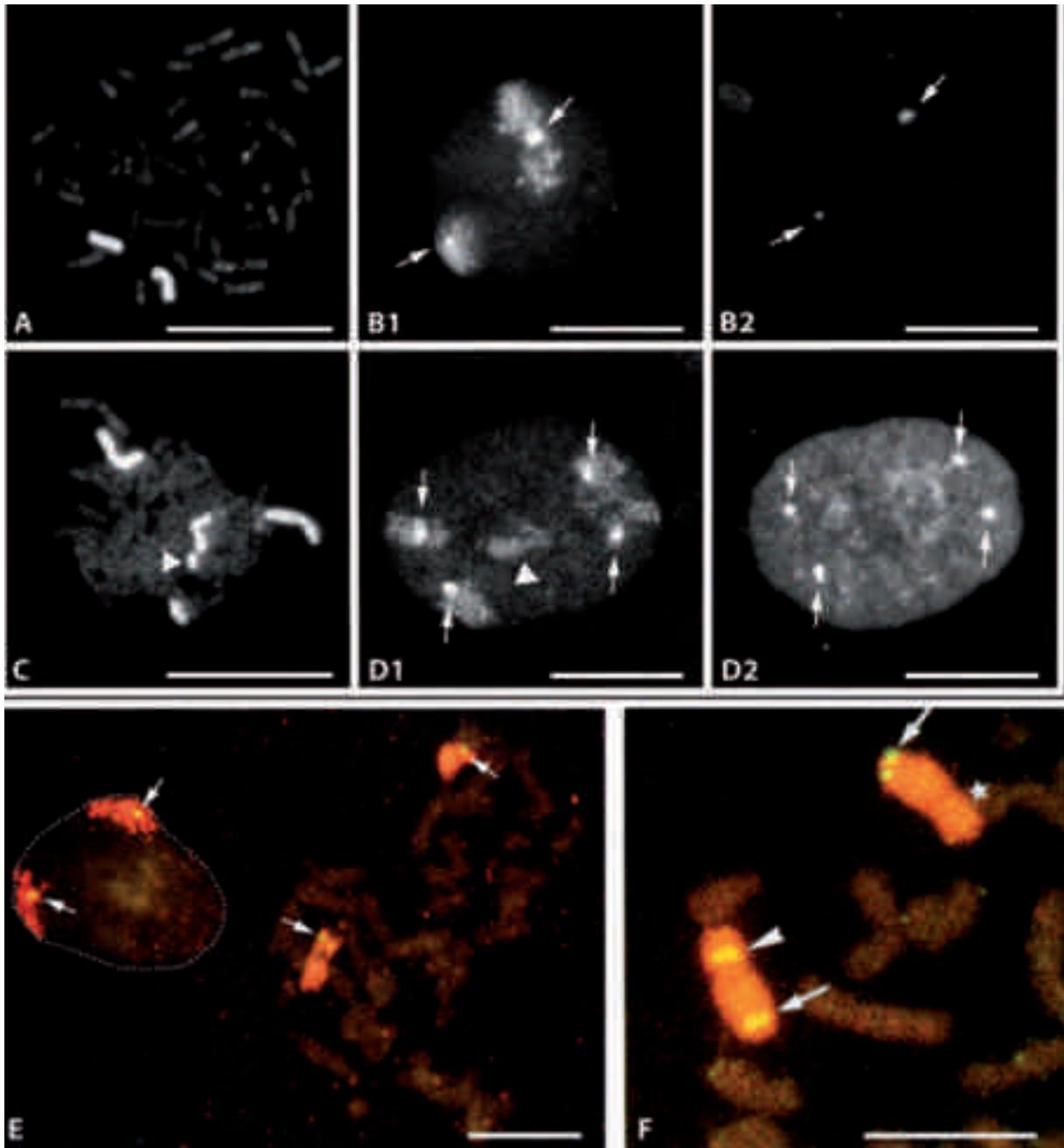
Figure 19. Chromomal *in situ* suppression (CISS) hybridization ("chromosome painting"): a tool to visualize individual chromosomes and chromosomal subregions at all stages of the cell cycle. A) Metaphase spread from a normal PHA-stimulated lymphocyte shows two painted chromosomes 7 following CISS hybridization with a HSA 7 specific library probe (Lichter *et al.,* 1988a). **B)** Interphase nucleus with two painted HSA 7 chromosome territories (B1). The HSA 7 pericentromeric heterochromatin was visualized by the simultaneous hybridization of a differentially labelled HSA 7 centromere specific alphoid DNA probe (B2) (compare arrows in B1 and B2) (Lichter *et al.,* 1988a). **C)** Metaphase spread from a cultured glioblastoma cell (C,D) shows an aberrant number of painted HSA 7 chromosomes (Cremer *et al.,* 1988b). **D)** Nucleus of a glioblastoma cell during interphase demonstrates five territories painted with the HSA 7 specific library probe. Four territories carry HSA 7 pericentromeric heterochromatin (D1, D2, arrows). The arrow-head points to the fifth territory lacking this region (reprinted from Cremer T., Lichter P. *et al.,* 1988b with permission). **E)** Two-color CISS hybridization of a diploid human fibroblast nucleus (46,XX) with a human X specific paint probe and a cosmid probe specific for the dystrophine gene reveals two painted X-chromosome territories (red), each marked with a differently coloured signal from the dystrophine gene (yellow). **F)** Metaphase spread from a female carrier of Duchenne muscular dystrophy with a deletion in one dystrophin gene. Two-color FISH was performed using three probes: the X-specific paint probe (red), a cosmid probe for the dystrophin gene (Xp21), which defines a region deleted in her son suffering from Duchenne muscular dystrophy (yellow). A second cosmid probe (also yellow), which maps to Xq28, was included as an internal control for hybridization efficiency. As expected one X lacks the hybridization signal on Xp21 (marked by asterix), while both X chromosomes show a signal at Xq28. This pattern was consistently observed in numerous metaphase spreads proving the carrier state of the mother and excluding a *de novo* deletion in the affected boy (Fig. E and F: unpublished example provided by Marion Cremer and Anna Jauch, 1994; for details, see Tocharoentanaphol *et al.* (1994). Bars correspond to 10 µm.

approach has also its limitations. It cannot be employed for studies of living cells and it lacks the possibilities of fluorescence microscopy to stain different structures with different fluorochromes or fluorochrome combinations.

Lacking personal experience with the dark sites and failures of scientific careers, we considered it simply fun in the early 1970s to think together about long term scientific projects none of us could have imagined alone and dismissed well meant advice that young wannabe scientists without a doctorate should better focus on experimental goals, which can be realized within two to three years. One of our dreams regarded the feasibility of a light microscope for three-dimensional (3D) studies of fluorescent biological specimens with a resolution beyond the Abbe limit. In 1970 we wrote a manuscript, where we described experiments of thought for the ultra-focusing of light (Cremer and Cremer, 1970). While the Abbe limit of structural resolution clearly holds for any conventional light microscope, we reasoned that it should be possible to break this limit by non-conventional means of focusing laser light. The smaller the focus, the better the optical resolution should become, when an object is scanned by a sharply focused laser beam. While the principle of scanning microscopy was already well established at this time using conventional microscope objectives (Minsky, 1956), we wanted to go further. The basic idea, which seemed physically feasible to us, was the generation of a three-dimensionally shaped hologram from an object with dimensions much smaller than the wavelength, called a 4π point hologram. (Note: $4\pi r^2$ gives the surface of a sphere with radius r; thus 4π suggests that a spherically shaped hologram recorded from a point like object emitting visible light should provide a means to reconstruct this object using laser light with appropriate intensities and phases from all sides). We reasoned that such a hologram could be used instead of conventional lenses to focus visible light down to a focal point much smaller than possible with any optical lens (Figure 20A). An object of interest could then be scanned point-by-point: Light emitted from molecules located in the focal point could be registered point by point with the help of a sensitive photomultiplier. Finally, the image of the entire scanned object could be composed electronically from the individual signals. We called this vision of an instrument a HoFo scanning microscope (HoFo = holo-

graphic focusing) (Figure 20B). As other potential applications of 4π point holograms we considered their use of in the context of computer data storage units or for the generation of extremely hot microplasms. When we sent our manuscript to a well-known professor of physics, he answered that he could not figure out what was wrong with our concept of holographic focusing, but that something certainly was wrong "because the [Abbe] resolution limit cannot be overcome". By chance we made the acquaintance of the patent attorney Ernst Sommerfeld (1899-1976), the son of the famous physicist Arnold Sommerfeld (1968-1951). Ernst Sommerfeld was more encouraging and kindly helped us to write a patent specification with our ideas. This specification was submitted to the German patent office in April 1971 together with our unpublished manuscript as an appendix. A disclosure of our patent claims was published in 1972 (Cremer and Cremer, 1972). Recent numerical calculations based on a theoretical approach proposed by Richard Feynman (Feynman, 1985) indicate that holographic focussing would indeed result in a substantial increase of the optical resolution beyond the Abbe limit (J. von Hase, C. Cremer, unpublished results). Although holographic focusing has not been realized to date, theoretical and technical advancements suggest that the replacement of conventional optical lenses by means of holographic focusing is still a valid concept.

The contribution of out of focus light results in smeared images and is a major disadvantage of conventional epifluorescence microscopy. Confocal imaging, first proposed by Minsky (1956), provided a possibility to overcome this problem (for a review of the history of confocal scanning microscopy see Inoué (2006). Based on our experience in focussing laser light close to the possible limit, we considered possibilities to develop our laser uv-microbeam apparatus further into a confocal laser scanning fluorescence microscope (compare Figure 6 with Figure 20C) (Cremer and Cremer, 1978) that would allow the generation of well resolved, three-dimensional images from single cells. Compared to other confocal microscopy designs (Brakenhoff *et al.,* 1979; Minsky, 1956), the innovative features of our design were a) to stain structures of interest within a biological specimen with fluorochromes and use laser light focussed to the diffraction limit with conventional optics to illuminate these structures point by point in three dimensions; b) to
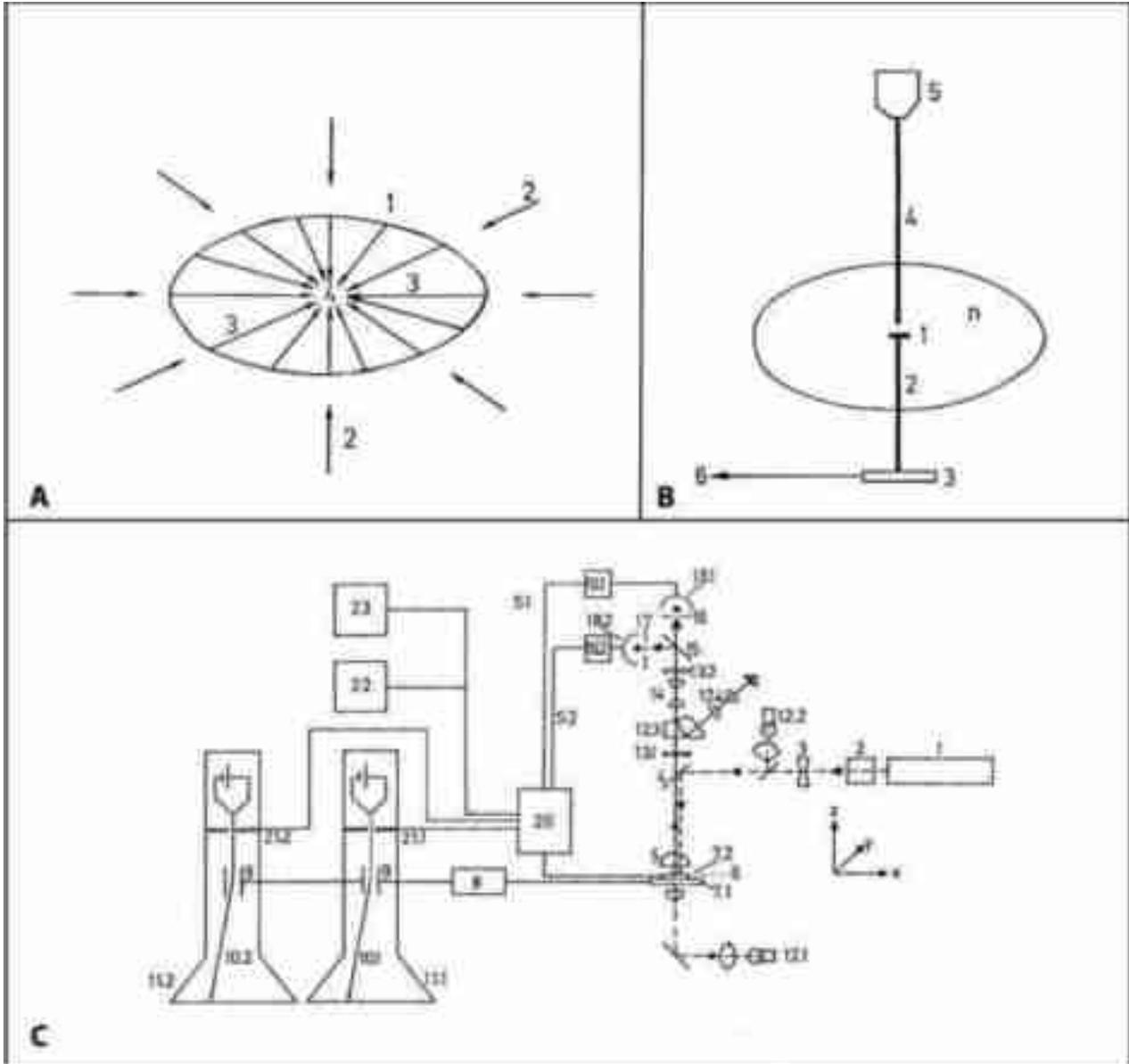
Figure 20. Various designs of laser scanning fluorescence microscopes. A) Focusing by a 4π-point-hologram (principle) (Figure 2a reprinted from Cremer C. and Cremer T., 1978, compare Cremer C. and Cremer., T 1971). 1) surface of the 4π-point-hologram (ideally a closed envelope); 2) incident waves; 3) reconstructed waves; 4) "focus" of the 4π-point-hologram. **B)** Application of a 4π-point-hologram instead of optical lenses in a [confocal] laser-scanning fluorescence microscope (Figure 2b reprinted from Cremer C. and Cremer T., 1978; see also Cremer C. and Cremer T., (1971): 1) specimen with a fluorescent surface located in the focus of a 4π-point-hologram; 2) holding device (same refractive index n as the specimen and the immersion fluid); 3) scanning stage to displace the specimen; 4) light conductor; 5) microscopic objective for collecting of the fluorescent light; detection system as in c); 6) connection to the scan generator. **C)** Laser scanning fluorescence microscope with conventional optics for the confocal ("point-by-point") analysis of three dimensional objects (reprinted from Cremer C. and Cremer T., 1978 with permission). 1) Laser system (continuous wave laser); 2) electro-optic modulator; 3) adjusting lens; 4) selecting mirror; 5) microscope objective for focusing the laser beam and for collecting the fluorescent light; 6) object plane; 7) scanning stage; 7.1 and 7.2) mechanisms for horizontal and vertical displacements of the object; 8) scan generator; 9) deflecting systems; 10) recording beams; 10.1 and 11.1) recording beam and TV-screen for topographical display of the specimen surface; 10.2 and 11.2) recording beam and TV-screen for display of the fluorescence distribution; 12) optical system for visual observation; 12.1 and 12.2) systems for transmitted and incident light illumination; 12.3) beam splitter; 12.4) eye piece; 13.1) barrier filter for elimination of the exciting laser light (wavelength λE); 13.2) narrow band filter for selection of fluorescence light (wavelength λF1); 14) magnifying system; 15) beam splitter (50%); 16) circular measuring diaphragm (image plane B1); 17) annular measuring diaphragm (image plane B2); 18.1 and 18.2) photomultipiers for measuring the luminous flux θ1 in the circular measuring diaphragms 16 (signal S1) and 17 (signal S2), respectively; 19.1 and 19.2) amplifiers for photomultipliers 18.1 and 18.2; 20) electronics of the automated focusing system; 21.1 and 21.2) intensity controls for recording beams 10.1 and 10.2; 22) facility for the two-dimensional numerical display of the specimen surface and of the fluorescent distribution, respectively (facultative); 23) facility for the three-dimensional display of the specimen surface and of the fluorescent distribution, respectively (facultative).

record the fluorescence emitted from each focal point using a detection pinhole to single out the main maximum of the diffraction image of the fluorescence signal of the excited object "point". Our design plan included the implementation of only a detection pinhole, while an excitation pinhole was avoided. This construction principle has been realized in confocal laser scanning microscopes sold by the Zeiss company. For lack of funding, however, we were not able to pursue the practical realization of this concept. In an appendix to our 1978 publication we outlined the idea of holographic focusing and of a radically new microscope type based on it.

In 1983 David A. Agard and John W. Sedat published a seminal paper about the three-dimensional topography of fluorescently stained polytene chromosomes in *Drosophila* nuclei using optical fluorescence microscopy and newly developed cellular image reconstruction techniques (Agard and Sedat, 1983). In the mid-1980s the first prototypes of confocal laser scanning fluorescence microscopes became available due to the independent, pioneering efforts of other groups. One prototype was built at the University of Amsterdam (Brakenhoff *et al.,* 1985; van der Voort and Brakenhoff, 1990) and another at the EMBL Heidelberg (Stelzer *et al.,* 1991; Wijnaendts-van-Resandt *et al.,* 1985) in collaboration with Josef Bille from the Physics Department of Heidelberg University. Compared to conventional epifluoresence microscopes, these instruments allowed light optical serial sectioning of cell nuclei with highly improved quality and yielded the possibility to study the three-dimensional nuclear distribution of painted CTs (Figure 21).

Breaking the Abbe limit of conventional light microscopy: witnessing an optical revolution in progress

While the classical resolution limit, which Ernst Abbe defined for classical light microscopy, holds true, even this genius could hardly dream of the present possibilities to circumvent this classical limit. We are presently witnessing an optical revolution in progress with profound consequences for cytology and histology. For a comprehensive, up-to-date account of these developments see (Pawley, 2006). This revolution will diminish the resolution gap between fluorescence and electron microscopy with the added advantage that fluorescence microscopy is suitable for studies not only of fixed but also of living cells. The wavelength of visible light per se is no longer considered a decisive limit for the best possible accuracy in the 3D localization of fluorescent targets or the maximum resolution of objects with details much smaller than the wavelength. The principle limit is given by the photon statistics. To put this difficult problem in plain language: in order to get an image from a given object with a resolution beyond the classical Abbe limit, the object must emit a sufficient number of photons. If the object moves during the time of recording, this number must be sufficiently large to produce a high-resolution image within a time period small enough to avoid image blurring.

Approaching the nanometer scale in high precision distance measurements between individual fluorescent targets

Precision distance microscopy (SPDM) (Bornfleth *et al.,* 1998; Heilemann *et al.,* 2002) makes use of the well established fact that the three-dimensional position of a 'point like' target, characterized by a given "spectral signature" (e.g. by a given fluorescence emission spectrum and/or fluorescence life time) can be measured much more precisely than the classical Abbe limit of resolution for light of the same wavelength. Distances between two targets emitting light of the same spectral signature, however, can be measured only as long as the distance between them is larger than the optical resolution. The development of SPDM was founded on the idea that in any type of fluorescence light microscope far smaller distances than the optical resolution of this microscope should become measurable, when the following conditions are fulfilled: a) the neighboring targets are characterized by different spectral signatures, which can be recorded independently, and b) the optical aberrations induced by the use of different spectral signatures can be precisely corrected. The principle limit of SPDM with regard to the localization precision of individual fluorophores is not determined by the wavelength of the emitted light, but by the size of the fluorophore and the number of photons, which can be recorded from an individual fluorophore (photon statistics). At high numbers of registered fluorescence photons, localization precision in the 0.2 nm range may be achieved (Heintzmann *et al.,* 1997). Although a localization precision for a given point-like target (e.g. a single molecule) in the order of a few nanometer is practically feasible at present, it does not help to distinguish two fluores-
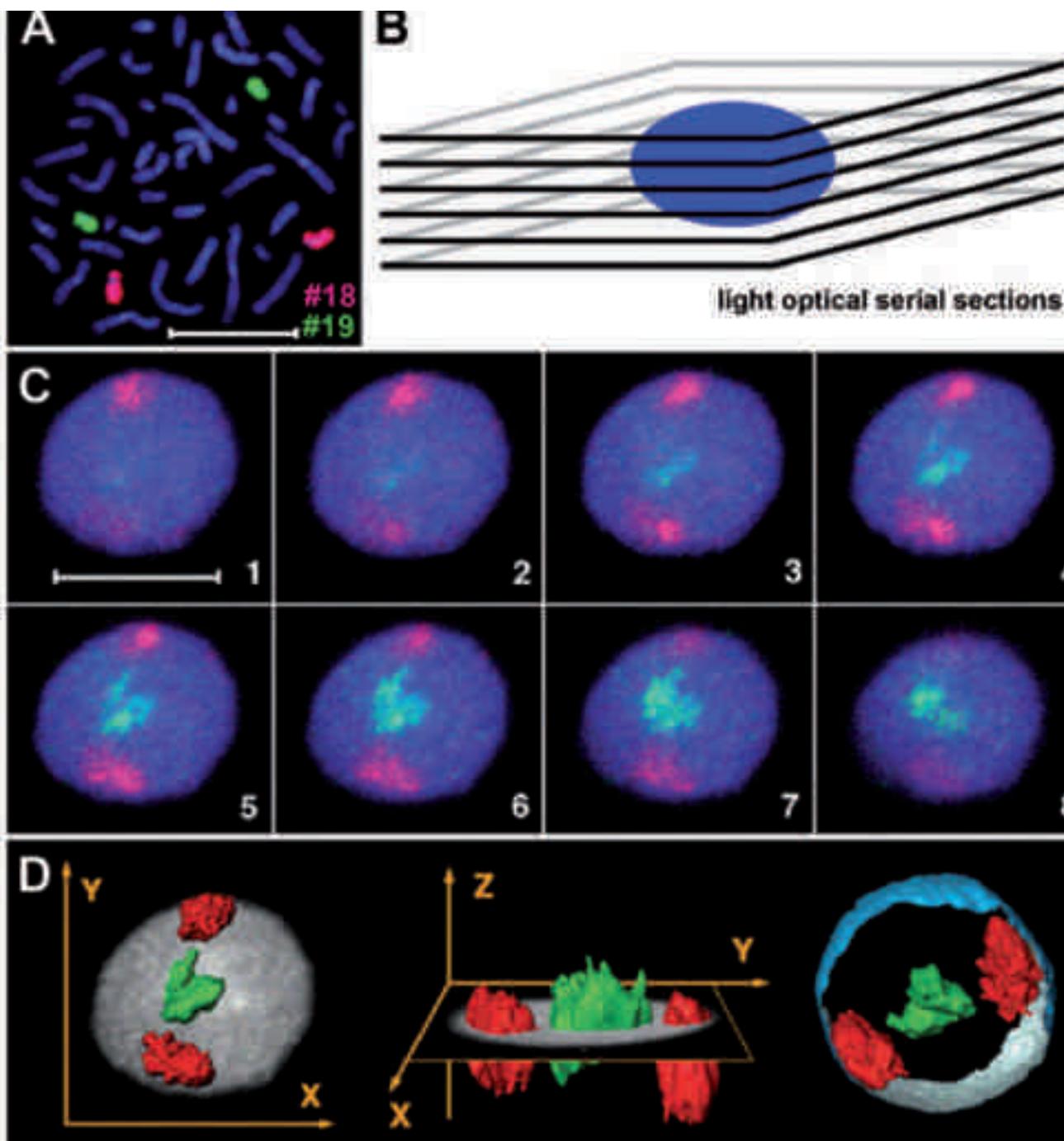
Figure 21. Light optical serial sectioning and 3D reconstruction of a human lymphocyte nucleus with differentially painted chromosome 18 and 19 territories. A) Two colour painting of chromosomes 18 (red) and 19 (green) in the metaphase spread of a diploid, PHA stimulated human lymphocyte. Chromosomes were counterstained with DAPI (blue). B) Scheme of light optical serial sections. Only a few sections are indicated. The total number of serial sections obtained from a spherically shaped lymphocyte nucleus typically varies between 30 and 50 sections. C) Eight light optical, nuclear sections selected from sites close to the nuclear top down to the nuclear bottom reveal the positions of the chromosome 18 (red) and 19 territories (green). DNA was counterstained with TOPRO-3 (false coloured in blue). D) 3D-reconstructions of the painted chromosome territories from the nucleus shown above viewed from different sites. The TOPRO-3 stained DNA from a single section is shown in gray colour in the left and middle figure. The figure on the right side shows the 3D reconstructed nuclear border (blue outside, gray inside) in addition to the territories. Note that HSA 19 territories (green) are typically located in the interior of human lymphocyte nuclei, whereas HSA 18 territories (red) are located at the nuclear periphery. Bars correspond to 10 µm.

cent point-like targets of the same colour in the object plane, which are closer to each other than the Abbe limit, since the diffraction images of these signals cannot be separated in the image plane. Labelling of different point-like targets with different fluorochromes, however, provides the possibility to exclude the contribution of the other differentially labelled target to the image by use of an appropriate filter. Since chromatic aberrations of light of different wavelength can be very precisely determined, the 3D positions of the intensity gravity centres of each target can be separately determined with a precision much smaller than the wavelength of the emitted light, even when these targets are located considerably closer to each other than the classical Abbe limit of the emitted peak fluorescence recorded from each target. Accordingly, SPDM has made it possible to perform precise 3D distance measurements as small as 50 nm between differentially labelled DNA sequences in single interphase nuclei (Esa *et al.,* 2000). In addition, using single molecules, SPDM distance measurements down to the 20 nm range in the object plane were realized (Heilemann *et al.,* 2002). Simultaneously, this method allowed the determination of the 3D positions of the individual targets with at least the same precision.

Approaching the nanometer scale in size measurements of submicroscopic fluorescent structures

Spatially modulated illumination (SMI) microscopy provides another tool to measure distances at the nanometer scale between two point-like targets along the optical axis. In addition, this approach has made it possible for the first time to measure fluorescently labelled structures with diameters much smaller than the classical Abbe limit. Under well defined optical conditions, the smallest, measured size was in the 40 nm diameter range (Failla *et al.,* 2002b), but the theoretical size limit was calculated to be considerably lower (Failla *et al.,* 2002b). Within intact cells fluorescently labelled complexes with diameters down to about 70 nanometer could be measured so far (Hildenbrand *et al.,* 2005; Martin *et al.,* 2004). SMI microscopy has already allowed measurements of the size range of transcription complexes (Martin *et al.,* 2004) and of small chromatin domains (Mathee *et al.,* 2006), including the approximate size of individual gene regions

(Hildenbrand, 2005). Since SMI microscopy allows to register 3D images of about 50 x 50 x 5 μm^3 in x,y,z within a few seconds, it can be used to measure the sizes of many thousands of intracellular macromolecular complexes within a reasonable time frame. In this way significant differences with errors of the mean in the very low nm range can be measured (D. Baddeley, C. Cardoso, C. Cremer, manuscript in preparation). Eventually, it should become possible to determine size differences of a given macromolecular complex resulting from the the binding or of loss of single proteins (D. Baddeley, C. Cardoso, C. Cremer, unpublished data).

Improving the resolution of fluorescence microscopy beyond the classical Abbe limit

It is important to distinguish between the possible precision with which the 3D localization of point-like, fluorescent targets (or of the intensity gravity centres of arger fluorescent objects, such as CTs) can be determined and the optical resolution of an entire image of a complex fluorescent structure. In confocal laser scanning fluorescence microscopy the image is obtained by illuminating excitable structures within the cell of interest 'point-by-point' and recording their fluorescence 'point-by-point'. The minimum distance between two excitable structures, which still allows the separate registration o the signals, depends on the focal diameter of the laser beam. During the 1990s Stefan Hell and colleagues pioneered the development of constructive focusing with an increased, effective numerical aperture by the use of two opposing conventional high numerical aperture lenses. Although the aperture angle achieved in such a ''4Pi'' laser confocal scanning microscope was only an approximation towards our goal of holographic focusing (*see above*), it was considerably higher than in any light microscope realized before and allowed a substantial improvement in axial resolution down to the 100 nm range using visible/near infrared light (Hänninen *et al.,* 1995; Hell *et al.,* 1994a). Since then 4Pi microscopy has been firmly established in cellular bioimaging (Baddeley *et al.,* 2006; Egner *et al.,* 2002; Egner *et al.,* 2004).

Another important advancement pioneered by Stefan Hell and colleagues, called stimulated emission depletion (STED) microscopy, has made it possible to overcome the classical Abbe limit both in axial direction and in the direction of the object plane. STED microscopy allows to decrease the

part of a given fluorescent structure, from which fluorescence can be recorded separately and thus the effective optical resolution down to the 15 to 20 nanometer scale (for review see Donnert *et al.*, 2006; Egner and Hell, 2005). The principal idea of STED microscopy is the following (Hell and Wichmann, 1994c; Schrader *et al.*, 1995): firstly, a small area (e.g. of 200 nm diameter) is excited by a first laser beam, and secondly, the fluorescence in this area is depleted by a second 'STED' laser beam (using a slightly other wavelength) except a very narrow central area (e.g. of 20 nm diameter). Under these conditions, only this tiny center will emit fluorescence photons of a given energy (wavelength) and thus be registered. The optical resolution (smallest distance resolvable) corresponds to the diameter of the tiny fluorescent center created by the STED process. Point by point scanning is realized with the help of piezoelectric elements, which allow movements of the object stage with nanometer precision. In the end the image can be reconstructed from the site dependent fluorescence intensities as in conventional laser scanning microscopy.

Patterned excitation microscopy (Gustafsson, 2000; Gustafsson, 2005; Gustafsson *et al.*, 1999; Heintzmann *et al.*, 2002) provides another way to overcome the classical Abbe limit of light microscopy. Most recently (Betzig *et al.*, 2006; Hess *et al.*, 2006) published a method called "photoactivated localization microscopy" (PALM), which allows an effective optical resolution in the 10 to 20 nm range even in the case that all objects have the same spectral signature. Instead of spatial scanning of the object, this localization microscopy approach requires the registration of multiple (thousands) of images of the same specimen. If the smallest size of photoswitchable fluorophores (Hofmann *et al.*, 2005) is assumed to be in the 1-2 nm range, then PALM should in principle allow to realize an effective optical resolution at the molecular level.

Commercial instruments realizing, e.g., the 4 Pi approach have already become available. Other commercial instruments, e.g., for STED or structured illumination microscopy, will soon follow. While it will still take some time to further develop these methods to the point, where they will be useful for routine applications in biological and medical research, each of the new methods will have its particular advantages and disadvantages. For example,

the time to record ultra-high resolution images with PALM is still extremely long (many hours), making this method presently suitable for a single image from a fixed specimen, but unsuitable for live cell imaging. To which extent faster PALM procedures can be established remains to be seen, although it may not be impossible to reduce the time finally required for a PALM image down to a few minutes, if not seconds. At its present stage of development, however, PALM cannot compete with STED microscopy and other approaches for applications in live cell imaging.

An unsurmountable limit for all electron microscopic approaches stems from the impossibility to study living cells. This is the major reason that the emerging field of live cell microscopy combined with the possibilities to circumvent the classical Abbe limit will have strong impact on the future of cell biology, which can hardly be overestimated. Yet, it would be a mistake to disregard the possibilities of the large spectrum of electron microscopic approaches. What matters is the ideal combination of advanced light and electron microscopic methods to solve particular imaging problems in cell biology. When applied in sequence to the same cell, serial sections performed first at light microscopic and thereafter at the electron microscopic level allow precise overlays of sections (Solovei *et al.*, 2002). 3D reconstructions, which take advantage of both light and electron microscopic serial sections will bring together the benefits of fluorescence microscopy to distinguish different structures by their specific staining with different colours with the highest possible structural resolution achieved by electron microscopy.

We conclude this paragraph by just mentioning a few other highlights, which are of particular interest for live cell imaging. Fluorescence recovery after photobleaching (FRAP) together with the invention of fusion proteins, which combine a protein of interest with green fluorescent protein (GFP) or a variety of other fluorescent proteins has revolutionized our understanding of the dynamics of protein interactions in the living cell (Misteli, 2001; Phair *et al.*, 2004). The use of fluorochromes with different fluorescence lifetimes in fluorescence lifetime imaging (FLIM) has increased the number of targets that can be simultaneously discriminated in single-cell studies (Schönle *et al.*, 2000). Fluorescence-resonance energy-transfer (FRET) microscopy has made it possible to study macromolecule interac-

tions in living-cells at interaction distances between ca. 2 and 8 nm (Damelin and Silver, 2000). Sub-micrometre particle tracking has been used to directly measure the motion of interphase chromatin (Bacher *et al.*, 2004; Bornfleth *et al.*, 1999; Chubb *et al.*, 2002; Gasser, 2002; Marshall *et al.*, 1997; Sage *et al.*, 2005). Advances in fluorescence correlation spectroscopy (FCS) have made possible detailed studies of diffusion-like processes of single molecules in living cells (Baudendistel *et al.*, 2005; Politz *et al.*, 1998; Wachsmuth *et al.*, 2003; Weidemann *et al.*, 2003). Last not least, the development of methods for the tracking of single fluorescent molecules in living cells has opened new avenues to test biophysical models of the functional nuclear architecture, which could not be tested before by biochemical and conventional ultra-structural approaches alone. Examples include experiments to pursue the pathway of single viruses labelled by a single fluorochrome into a cell and its nucleus (Brauchle *et al.*, 2002; Seisenberger *et al.*, 2001) and of single molecules passing through the nuclear pore complex (Kubitscheck *et al.*, 2005).

Part III. Chromosome territories and the functional nuclear architecture

The biological goal of our research efforts during the last thirty years was to develop and apply a combination of methods, which should help to elucidate principles of the nuclear architecture based on comparative studies of nuclear phenotypes as outlined in the review from 1987 (Cremer *et al.*, 1987) (see introduction to part II). The state of progress in the field of nuclear archtitecture in 1990 was described in a seminal review from Laura Manuelidis (Manuelidis, 1990), a pioneer of modern research on nuclear architecture (Borden and Manuelidis, 1988; Manuelidis, 1984a; Manuelidis, 1984b; Manuelidis, 1985; Manuelidis, 1985a; Manuelidis and Borden, 1988)

The results of experimental work performed by us and others since the 1990s have been summarized in numerous reviews and books (Berezney, 2002; Chevret *et al.*, 2000; Cook, 2001; Cook *et al.*, 1996; Cremer and Cremer, 2001a; Cremer *et al.*, 2006a; Cremer *et al.*, 2000; Cremer *et al.*, 1993; Feuerbach *et al.*, 2002; Foster and Bridger, 2005; Hemmerich and Diekmann, 2005; Kosak and Groudine, 2004; Lanctot *et al.*, 2006; Marshall, 2003; Marshall and Sedat, 1999; Misteli, 2004; O'Brien *et al.*, 2003; Parada *et al.*, 2004; Park and

De Boni, 1999; Pederson, 2004; Politz *et al.*, 2003; van Driel and Fransz, 2004; van Driel *et al.*, 2003; Zink *et al.*, 2004). For space limitations we decided to restrict this review to work exploring the nuclear architecture of vertebrates, but wish to emphasize here at least in passing the groundbreaking research on the nuclear architecture in Drosophila and yeast (Agard and Sedat, 1983; Gasser, 2002; Marshall *et al.*, 1996; Vazquez *et al.*, 2001) and plant species (Abranches *et al.*, 1998; Heslop-Harrison, 2003; Lysak *et al.*, 2003; Pecinka *et al.*, 2004). These studies demonstrated a territorial organization of chromosomes in both animal and plant species. They also demonstrated similarities, as well as distinct differences of CT arrangements both between different cell types (e.g. lymphocytes vs. fibroblasts) of the same species and between the same cell type studied in different species. In spherically shaped nuclei of human lymphocytes or lymphoblastoid cells gene density correlated radial nuclear arrangements of CTs were observed. Gene dense CTs, such as HSA 19 CTs, are located in a more central location, while gene poor CTs, such as HSA 18 CTs are positioned at the nuclear periphery (Boyle *et al.*, 2001; Cremer and Cremer, 2001a). In contrast, chromosome size in addition to gene density affects the positioning of CTs in flat-ellipsoidal nuclei of human fibroblasts (Bolzer *et al.*, 2005; Croft *et al.*, 1999). In 2D-projections CTs of large chromosomes were preferentially detected at the nuclear equator and small CTs close to the nuclear centre. In axial direction, however, an impact of gene density was noted. HSA 18 CTs were generally found broadly attached to the upper or lower part of the nuclear envelope in contrast to the more remote location of HSA 19 CTs (Bolzer *et al.*, 2005). In this study we employed for the first time the possibility to distinguish all 23 human chromosome types in single diploid fibroblast nuclei from a male human being (Bolzer *et al.*, 2005; Schrock *et al.*, 1996; Speicher *et al.*, 1996). It is less clear at present to which extent non-random neighbourhoods of CTs and/or of chromosomal subregions exist in human and other mammalian cell types. In human lymphocyte and fibroblast cells we observed highly variable neighbourhood arrangements of both CTs and prometaphase chromosomes (Bolzer *et al.*, 2005). This variability was confirmed in small cell clones of normal diploid human cell cultures and tumour cell lines (unpublished data) and defies previous claims of a highly

defined order (Bolzer *et al.*, 2005; Nagele *et al.*, 1995). The question, which biological parameters of chromosomes, such as chromosome size, replication timing, gene density and expression levels, correlate best with (or may even cause) the non-random radial nuclear positioning of CTs has not been settled so far. G- and R-bands of mammalian metaphase chromosomes are characterized by profound differences in gene density, GC content, replication timing and chromatin compaction. Although the preferential localization of gene dense, transcriptionally active and early replicating chromatin in the nuclear interior and of gene poor, later replicating chromatin at the nuclear envelope has been demonstrated to be evolutionary conserved, it is not known whether radial chromatin positioning is preferentially shaped by local gene density per se or by other related parameters, such as replication timing or transcriptional activity. The interdependence of these distinct chromatin features on the linear DNA sequence precludes a simple dissection of these parameters with respect to their importance for the reorganization of the linear DNA organization into the distinct radial chromatin arrangements observed in the nuclear space. Gene density correlates with replication timing and gene dense regions may often contain many more transcriptionally active genes and produce more nascent RNA than gene poor regions. To analyze this problem, we generated probe-sets of pooled BAC clones from HSA 11, 12, 18 and 19 representing R/G-band assigned chromatin, segments with different gene density and gene loci with different expression levels (Kuepper *et al.*, 2006). Using multicolor 3D-FISH and 3D image analysis, we determined their localization in the nucleus, as well as their positions within or outside the corresponding chromosome territory (CT). For each BAC data on local gene density within 2 and 10 Mb windows, as well as GC content, replication timing and expression levels, were determined. A correlation analysis of these parameters with nuclear positioning revealed regional gene density as the decisive parameter determining the radial positioning of chromatin in the nucleus in contrast to band assignment, replication timing and transcriptional activity. We demonstrate a polarized distribution of gene-dense versus gene-poor chromatin within CTs with respect to the nuclear border. Whereas we could confirm previous reports that a gene-dense and transcriptionally highly active region of about 2 Mb on 11p15.5 can

loop out from the surface of the HSA 11 territory, its compaction level was much higher than expected for a 30 nm chromatin fiber. Furthermore, gene-dense and highly expressed sequences were not generally found preferentially at the CT surface as previously suggested (Kuepper *et al.*, 2006).

The dynamic topography of nuclear structures may add another level of complexity to gene regulation

Chromosome territories are complexly folded structures with genes with actively transcribed genes found both at the periphery and within the interior of CTs (Bartova *et al.*, 2002; Dundr and Misteli, 2001; Kuepper *et al.*, 2006; Mahy *et al.*, 2002b). The regulation of gene expression is mediated by interactions between chromatin and regulatory protein complexes. The importance of where and when these interactions take place in the nucleus is has become the subject of intense investigations and conflicting models (Cremer *et al.*, 2006a; Lanctot *et al.*, 2006; Williams, 2003). In addition to cell-type specific patterns of DNA methylation, histone modification and chromatin remodelling, which presently take centre stage in epigenetic research (Fuks, 2005; Jenuwein and Allis, 2001; Klose and Bird, 2006; Martin and Zhang, 2005; Mellor, 2005; Saha *et al.*, 2006) the dynamic topography of higher order chromatin domains, nuclear speckles and bodies, as well as the dynamic nature of the (re)positioning of genetic material in the nuclear space suggests an even higher level of epigenetic gene regulation. All these levels are likely integrated with each other to realize the epigenome of a given cell, which is responsible for the cell type specific orchestration of gene expression and other important nuclear functions, such as chromatin replication and the maintainance of genomic integrity. Recent evidence indicates that activation or silencing of specific genes may be associated with their repositioning relative to other genomic loci and nuclear compartments (for review see (Lanctot *et al.*, 2006). Fluorescent labeling of transgenes in living cells has provided opportunities to observe directly changes of chromatin condensation, as well as positional changes of such transgenes during activation or silencing in the nucleus of living cells (Janicki *et al.*, 2004; Robinett *et al.*, 1996). Whether such repositioning events imply unknown mechanisms for directed movements of chromatin or depend on Brownian movements is not known. In any case, structural constraints

impose limits on chromatin mobility in human and other mammalian cell nuclei (Abney *et al.*, 1997; Bornfleth *et al.*, 1999; Walter *et al.*, 2003), which in nuclei of yeast species may be of minor importance due to the much smaller size of these nuclei (Chubb and Bickmore, 2003; Gasser, 2002). Below we describe several models, which emphasize the range of present opinions about basic principles of the nuclear architecture and their relation to gene regulation and other nuclear functions. We present the evidence provided in favour of these models and attempt a critical appraisal of their pros and cons.

From the interchromosome domain model to the chromosome territory – interchromatin compartment model

In 1993 the interchromosome domain (ICD) model of nuclear architecture was published (Figure 22A). This model shared important features with a model provided at the same time by Bingham and colleagues for polytene nuclei of Drosophila (Kramer *et al.*, 1994; Zachar *et al.*, 1993). Both models argued for a subnuclear compartment consisting of a network of channels defined by exclusion from condensed higher order chromatin configurations. A first version of the ICD model (Zirbel *et al.*, 1993) considered CTs as objects with a rather smooth surface entirely separated from each other by an DNA free ICD. The ICD model revived previous models of Geitler (Geitler, 1943) (see Figure 9 in part I of this review) and of Pollister (Pollister, 1952) (Figure 1A), which were unknown to us in 1993. In contrast to these previous models, however, the ICD model argued for a functional role of the ICD.

The original version of the ICD model (Zirbel *et al.*, 1993) implied that (i) CTs have a rather smooth surface and are separated from each other by an interchromosome domain (ICD) running between the surfaces of CTs; (ii) active genes are located at the periphery of CTs in order to allow functional interactions with the constituents of the ICD, such as nuclear speckles (interchromatin granule clusters) and nuclear bodies. Subsequently, it was emphasized that the surface of chromosome territories can be greatly increased by infoldings (see magnified part of the model nucleus in Figure 22A) (Cremer T., Kurz A. *et al.*, 1993). These infoldings can account for the observation of active genes in the interior of CTs (Mahy *et al.*, 2002b). Later we coined the term interchromatin domain

compartment (Cremer T., Dietzel, S. *et al.*, 1995) or briefly interchromatin compartment (IC) (Cremer T. and Cremer C. , 2001a; Cremer T., Dietzel, S. *et al.*, 1995; Cremer T., Kreth, H. *et al.*, 2000) to emphasize our observation of a three-dimensional network of lacunas and channels with a width clearly detectable by light microscopic observations (Albiez *et al.*, 2006). This compartment (Figure 23A) starts at nuclear pores and runs both between CTs and into the interior of CTs and supposedly ends with its finest branches only detectable at the ultra-structural level between ~1 Mb chromatin domains or even smaller ~100 kb domains (Figure 23). The IC contains nuclear bodies, such as Cajal (coiled) bodies, previously called interchromatin granule clusters by electron microscopists. While these speckles are enriched in splicing factors, they are not sites of splicing (for review see (Fakan, 2004a; Fakan, 2004b).

Chromatin domains with a DNA content in the order of ~1 Mb were originally observed in mammalian cell nuclei as replication foci (Ma *et al.*, 1998; Nakamura *et al.*, 1986; Nakayasu and Berezney, 1989). Later it was shown that individual domains persist as higher order chromatin structures outside S-phase and most likely from one cell cycle to the next (Cremer and Cremer, 2001a; Walter *et al.*, 2003; Zink *et al.*, 1998). The internal structure of these domains is not known. We have proposed that each ~1-Mb chromatin domain is built up as a rosette of ~100-kb chromatin loop domains (Figure 23B,C) with anchoring proteins to tether ~100-kb domains together at the centre of ~1-Mb domains. Based on the finding that complete extraction of internal nuclear matrix components with RNase treatment followed by 2 M NaCl results in the disruption of higher order chromosome territory architecture (Ma *et al.*, 1999), suggested that a nuclear matrix, which includes a RNA component, helps to stabilize and maintain CTs and chromatin domains, which built up CTs.

Ultrastructural evidence for the IC stems from ultrathin sections of mouse pancreas cell nuclei specifically stained for DNA with a highly sensitive Feulgen-type procedure (Cogliati and Gautier, 1973)(for reviews see (Fakan, 2004a; Fakan, 2004b). About 40 to 50% of the nuclear volume was occupied by an interchromatin space mostly devoid of DNA. This space expanded between clumps of chromatin and did not include any space free of DNA within these chromatin clumps. Below
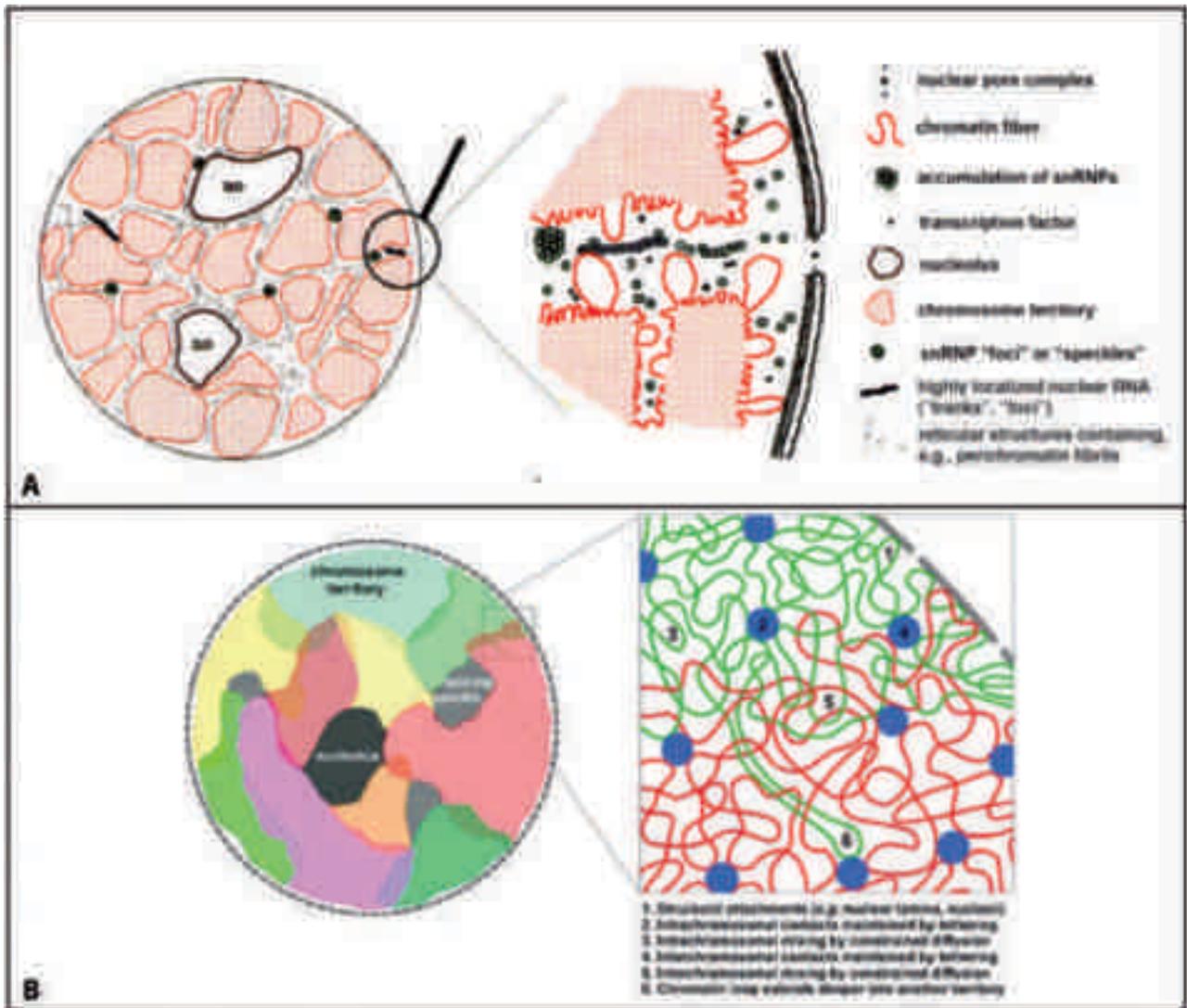
Figure 22. A) Interchromosomal domain (ICD) model. This model (reprinted from Cremer T., Kurz, A. *et al.,* 1993 with permission) was first described by Zirbel *et al.,* 1993. It argued for an interchromosomal domain (ICD) space, which surrounds individual CTs at all sites and thus separates them entirely from neighbouring CTs. The ICD contains functional machineries, such as transcription machineries, nuclear speckles and bodies (see magnified portion of the model). This magnified portion also emphasizes the possibility that chromatin loops expand from territory surfaces into the ICD space. While the original version from Zirbel *et al.,* 1993 took into account CTs with a rather smooth surface, Cremer T., Kurz, A. *et al.,* 1993 argued that "the surface of CTs can be greatly increased by infoldings". **B) Interchromosomal network (ICN) model** (reprinted from Branco and Pombo 2006 with permission), Branco and Pombo, 2006 argued that "chromatin from different chromosomes is not separated by a compartment but is allowed to expand into the surrounding territories; the presence of adjacent chromosomes, the nuclear membrane, and larger nuclear compartments restricts the amount of intermingling. DNA sequences along chromosomes will have different properties that determine their compaction, mobility, and affinity to specific nuclear components. Despite the different local levels of compaction, the global average properties of chromatin in areas of intermingling will be similar to those found within a CT. Rare chromatin loops extending from a CT can invade neighboring CTs."

we review two recently proposed models of the functional nuclear architecture, which deny the existence of both a functionally relevant interchromatin compartment and a perichromatin region.

The transient incubation of living cells in medium with increased osmolarity provides a means to manipulate the width of the IC without affecting the viability of cells (Albiez *et al.,* 2006). This procedure yielded the rapid formation of hypercondensed chromatin and the concomitant widening of the IC space. The effect was fully reversible, when the cells were again incubated in medium with normal osmolarity. Repeated cycles between normally condensed (NCC) and hypercondensed chromatin (HCC) yielded reproducible patterns of a contiguous HCC bundles together with a contiguous IC space. These reproducible patterns suggest a structural memory, whose molecular foundation is still unknown. Notably, cells retained their viability even after several NCC-HCC-NCC cycles. A quantitative analysis of the topological relationships of nuclear speckles, bodies, RNA polymerase II and nascent

RNA revealed the presence of these structures predominantly either within the expanded IC (nuclear speckles and bodies) or at the surface of hypercondensed chromatin bundles (RNA-polymerase II, nascent RNA).

Evidence for the perichromatin region as a nuclear compartment for transcription, splicing and DNA-replication

In two recent reviews Stanislav Fakan (Fakan, 2004a; Fakan, 2004b) has summarized electron microscopic studies performed by his own and others groups over the last 30 years. These studies have provided profound evidence for a specific nuclear compartment, called the perichromatin region, where transcription, splicing and DNA-replication takes place. The perichromatin region comprises a zone of decondensed chromatin, which is localized at the periphery of compact chromatin domains and separates these domains from the IC interior. Evidence for nascent RNA formation in the perichromatin region was originally obtained by the application of tritium labelled RNA precursors and high resolution autoradiography (Fakan and Bernhard, 1971) (see Introduction to part II) and later by non-radioactive labeling methods of RNA in combination with immunoelectron microscopy. In these studies perichromatin fibrils, first described by (Monneron and Bernhard, 1969), were identified as structures, which contain rapidly labeled RNA synthesized within the perichromatin region (Fakan et al., 1976; Nash et al., 1975) and represent in situ forms of hnRNA transcripts (Fakan, 1994). Immunoelectron microscopy further revealed the association of hnRNP core proteins and of transcription factors, such as RNA polymerase II and TFIIH, with perichromatin fibrils (Cmarko et al., 1999). Perichromatin fibrils are also associated with factors of pre-mRNA processing, including snRNPs, m3G cap structure of snRNAs, SC-35 protein, poly(A) polymerase and others (for details see (Fakan, 2004a; Fakan, 2004b)). Based on these obervations Fakan concluded that perichromatin fibrils are specific nuclear structures connected with transcription and co-transcriptional splicing. This conclusion implies that the majority of active genes or at least those parts actually being transcribed at any given moment should be located in this compartment. While the CT-IC model predicted that silent genes are located in the interior of compact chromatin domains, observations that polycomb group gene silencing proteins are concentrated in the perichromatin region suggest that epigenetically silenced genes may be localized in the perichromatin region (Cmarko et al., 2003).

The formation of a given perichromatin fibril depends on the transcription of a single gene, other authors reported transcription factories with about 50 nm diameter, which contain many RNA polymerases II and III, respectively, and are able to transcribe a number of genes simultaneously (Iborra et al., 1996; Jackson et al., 1998; Martin and Pombo, 2003). These transcription factories were implicated in cases of "gene kissing", i.e. the spatial co-localization of gene loci located on different chromosomes and of remote sites within a chromosome (Osborne et al., 2004), (Chakalova et al., 2005; Lanctot et al., 2006).

In addition to transcription and splicing events, the perichromatin region was also identified as the compartment where DNA is replicated. Early high resolution in situ studies of DNA replication were carried out using ^{3}H-thymidine as DNA precursor and EM autoradiography and indicated that most DNA replication sites, visualized after very short labelling pulses of exponentially growing cells occurred at the periphery of condensed chromatin areas (Fakan and Hancock, 1974). This conclusion was confirmed in later studies, which employed 2 min pulses with halogenated deoxyuridines and immunocytochemistry with colloidal gold markers (Jaunin et al., 2000). Nascent DNA co-localized with DNA polymerase, cyclin A and PCNA (Sobczak-Thepot et al., 1993). Movement of DNA into the interior of compact chromatin domains during the replication process was demonstrated in pulse-chase experiments, in which DNA replicated shortly before and after the chase was differentially visualized by the incorporation of two halogenated nucleotides (IdU and CldU) (Jaunin et al., 2000).

The Lattice model for the organization of chromatin in interphase cells

The concept of an interchromatin compartment mostly avoid of DNA, which is separated from compact chromatin domains by a perichromatin region has been challenged in a recent review from David Bazett-Jones and coworkers (Dehghani et al., 2005). These authors employed energy filtered transmission electron microscopy (EFTEM), also referred to as electron spectroscopic imaging (ESI), to study the organization of chromatin in the
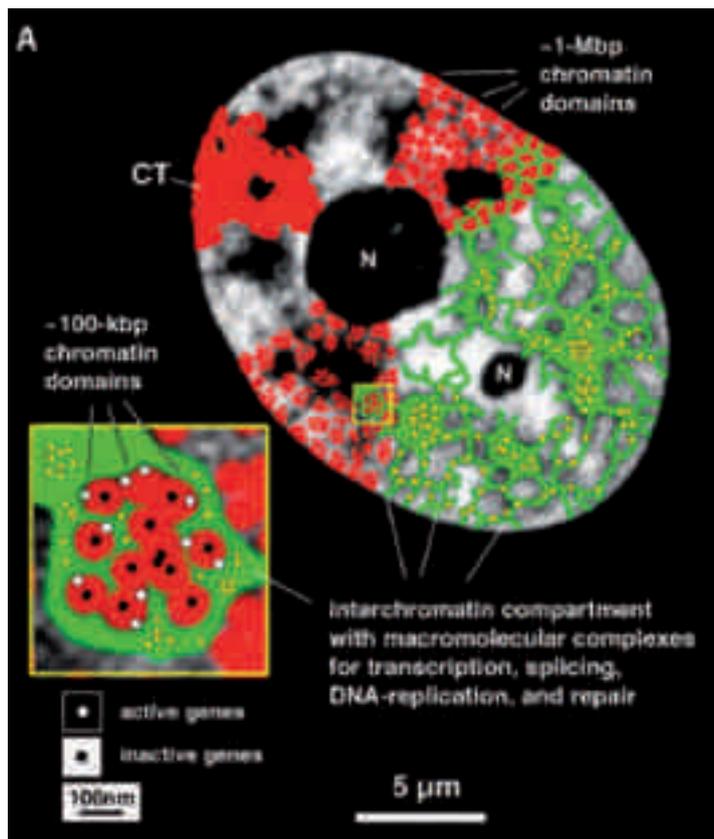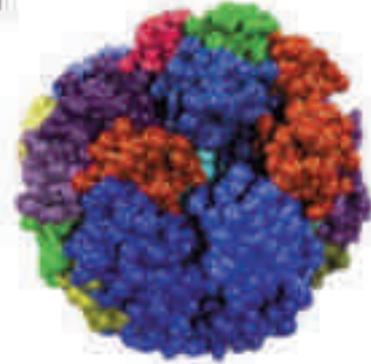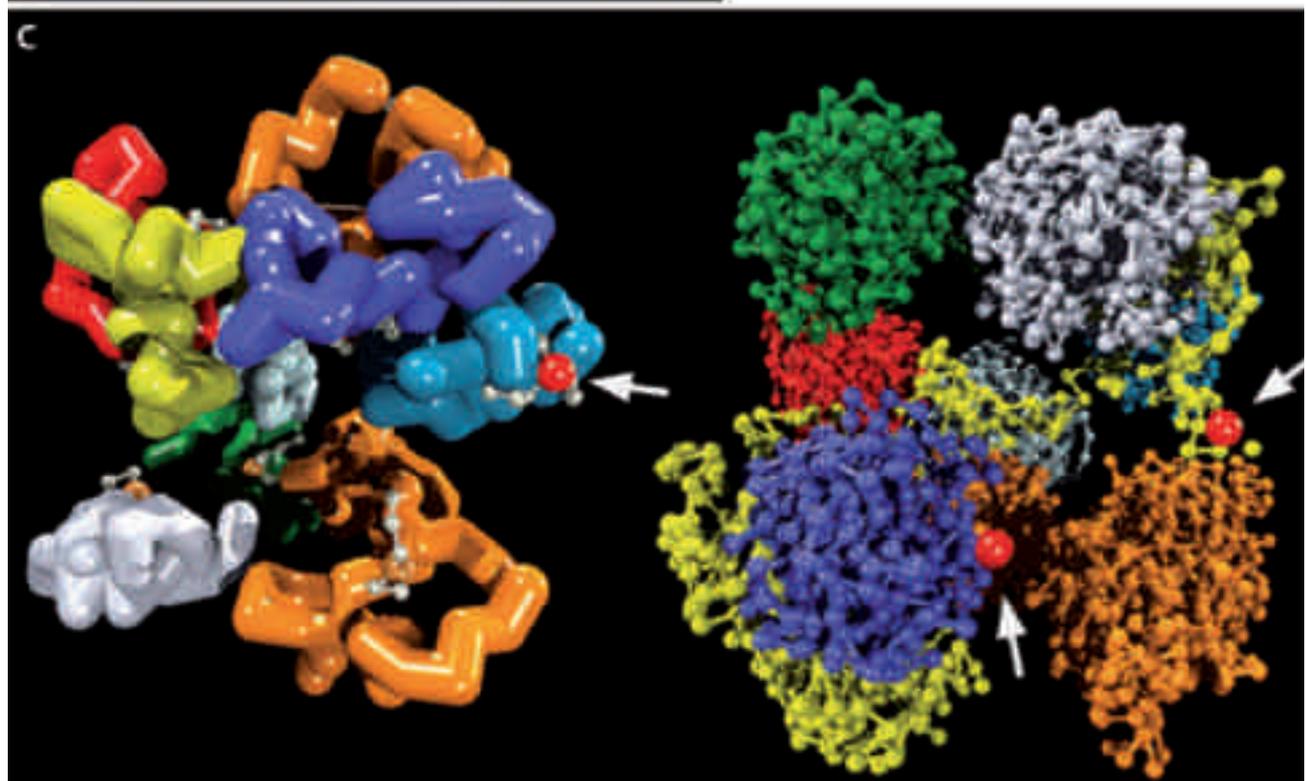
interphase nucleus. This method provides maps of specific elements, such as nitrogen and phosphorus. The authors did not detect a network of channels expanding between CTs and in the interior of CTs and concluded that chromatin the interphase nucleus of mammalian cells is organized as a meshwork or *lattice* of 10 and 30 nm thick chromatin fibres. A concern with the experimental basis of this *lattice* model results from the fact that ESI imaging of phosphorus does not distinguish between phosphorus from DNA and RNA. In addition, although the localization of proteins by ESI seems straight forward, heavily phophorylated proteins present in the interchromatin space may further complicate the interpretation of ESI images.

The authors emphasize a lack of specificity of the EDTA-regressive staining procedure introduced by (Bernhard, 1969) as a means to specifically contrast RNA and conclude that chromatin negatively contrasted by this technique represents condensed heterochromatin, predominantly clustered at the nuclear envelope and peripheral to nucleolar regions, while most euchromatin arguably escaped detection. According to (Dehghani *et al.*, 2005)

"perichromatin fibrils represent a subset of extranucleolar RNA transcripts that are peripheral to blocks of heterochromatin, but do not represent the transcripts that are synthesized throughout chromosome territories." (Dehghani *et al.*, 2005) emphasized the hazards of inferring chromatin ultrastructure from fluorescence microscopy and argue that fluorescence microscopic observations of higher order chromatin arrangements, such as ~1 Mb chromatin domains, are a misperception that arises in particular from the disparity in resolution between the light and electron microscope. Accordingly, what may appear as a ~1 Mb chromatin domain at the resolution of the confocal microscope may in fact represent "local concentrations of 30 and 10 nm fibers". Surprisingly, the authors do not discuss in this context the electron microscopic evidence described above in favour of chromatin domains separated by the IC using a sensitive Feulgen type staining method for DNA in ultrathin, nuclear sections nor the evidence that argues against the formation of nascent RNA in the interior of the IC.

Figure 23. (page 258) A) Chromosome territory-interchromatin compartment (CT-IC) model. The CT-IC model (Cremer C. and Cremer T., 2001a; Cremer T., Dietzel S. *et al.*, 1995; Cremer T., Kreth G. *et al.*, 2000) was developed on the basis of the ICD model (Figure 22A). The IC comprises a network of DNA channels/lacunas largely avoid of DNA. It expands from the nuclear pores into the nuclear interior both between neighbouring CTs and into the interior of CTs. Although IC channels/lacunas are found between neighbouring CTs, they do not separate them completely from each other as originally proposed by the ICD model. This aspect of the CT-IC model is consistent with electron microscopic evidence, which demonstrated that CTs form direct contacts with each other not separated by any space detectable at electron microscopic resolution (Visser *et al.*, 2000) and with recent evidence that CTs form a contiguous 3D network of higher order chromatin domains/bundles above the compaction level of 30 nm thick chromatin fibres (Albiez *et al.*, 2006). The insert shows a ~1 Mb chromatin domain built up from a series of ~100 kb loop domains in a compacted state. Genes poised for transcription are located at the surface of these ~100 kb domains, while silent genes are retracted into the domain interior. This scenario argues for gene repositioning during both transcriptional activation and silencing of genes at a scale that cannot be detected with conventional light optical imaging. B) Spherical ~1-Mb chromatin-domain (SCD) model. The SCD model was developed by Gregor Kreth and C. Cremer. Each spherical chromatin domain has a diameter of 500 nm and represents a ~1Mb chromatin domain. The model allows for slight volume overlaps between neighbouring SCDs (Cremer T., Kreth G. *et al.*, 2000; Kreth *et al.*, 2004a). G. Kreth deserves all credit for quantitative simulations based on the SCD model (Bolzer *et al.*, 2005) and for preparing the 3D model images. B1) SCD model of a male, diploid human cell nucleus with spherical shape and 46 independently distributed CTs (reprinted with permission from Cremer and Cremer, 2001a supporting online material). The 24 chromosomes types (22 autosomes, 1 X and 1 Y) are visualized using 24 pseudocolours. B2) Virtual midplane 'low resolution' ultrastructural sections through the SCD model nucleus shown in (B1) reveals an interchromatin compartment space of variable width expanding between the spherical chromatin domains. Note that in this grey-coloured, 'low resolution' virtual section individual CTs cannot be distinguished and that individual ~1-Mb chromatin-domains may be located so close to each other that the narrow interchromatin channel between them cannot be recognized or they may connect to each other to form higher order chromatin bundles (Albiez *et al.*, 2006). C) Two versions of a hypothetical ultrastructure of ~1-Mb chromatin domains modelled by G. Kreth (compare Munkel *et al.*, 1999) reprinted from Cremer T., Kreth G. *et al.*, 2000 with permission). The ~1-Mb chromatin domain model shown on the left side is built up from ten ~100-kb loop domains (Munkel *et al.*, 1999); domains visible from the given perspective are discriminated by different colours. A continuous 30-nm thick chromatin fibre represented by random walks of short cylinder segments is occasionally interrupted by short regions of 10 nm thick nucleosome chains (small white dots). In the ~1-Mb chromatin domain model shown on the right |each of the ten 100-kb chromatin domains was modelled under the assumption of a restricted random walk (zig-zag) nucleosome chain. Each dot represents an individual nucleosome. The ~100-kb chromatin domains are shown in a "closed" configuration except for the yellow coloured domain, which demonstrates a relaxed chain structure ("open" configuration) expanding at the periphery of the 1-Mb chromatin domain. This open domain will have enhanced accessibility to the interchromatin compartment and its content of individual transcription and splicing factors or factor complexes present in the IC. The arrows point to red spheres with a diameter of 30 nm, each representing a functional machinery, such as a transcription factor or splicing factor complex.

The interchromatin compartment network model

To circumvent the limited resolution of conventional laser confocal scanning microscopyy in axial direction (about 700 nm) (Branco and Pombo, 2006) developed a cryo-FISH procedure. Sucrose-embedded human peripheral blood lymphocytes were fixed under structure conserving conditions. Ultrathin cryosections (approximately 150 nm thick) were used for FISH with whole chromosome paint and sub-regional probes. The authors confirmed the presence of chromosome territories in interphase nuclei and demonstrated that CTs are not entirely separated from each other by an inter-chromosomal domain (ICD). They also describe - in agreement with the results of Deghani *et al.* (2005) but contradictory to the CT-IC model described above - that chromatin fibres intermingle profoundly in interphase nuclei of human cells both between neighbouring CTs and within CTs. Based on these findings, Branco and Pombo proposed the inter-chromatin compartment network (ICN) model (Figure 22B). In agreement with the *lattice* model from Bazett-Jones and co-workers the ICN model denies the existence of an interchromatin compartment and emphasizes the intermingling of chromatin loops both between neighbouring CTs and within the interior of individual CTs. Branco and Pombo (2006) argue that "the fraction of one chromosome (both homologs) that intermingles with any of the other 22 chromosomes is, on average, 2.1±1.1%." This value is in agreement with the order of intermingling predicted by the multi-loop subcompartment (MLS) model of chromosome territory organization (Munkel *et al.*, 1999; Münkel and Langowski, 1998) and experimental data for the overlap of chromosome 3 and chromosome 6 arm domains in human diploid amniotic fluid cell nuclei, which showed that noticeable inter-mingling of chromatin loops from these two arm was limited to a narrow boundary zone (Dietzel *et al.*, 1998). Branco and Pombo further concluded that 2.1% intermingling between two chromosomes "would correspond to 46% of each chromosome being intermingled with the rest of the genome (2.1% x 22 chromosomes)." The authors attempted the quantification of the total intermingling of the painted human chromosome 3 territory with the remaining genome, which was co-hybridized with a probe for all remaining human chromosomes, and found that 41% of the volume of chromosome 3 contains chromatin intermingled with other chromosomes. These values suggest to us that any CT should be profoundly invaded from chromatin fibres expanding from neighbouring CTs.

The experimental and theoretical support provided by Branco and Pombo (2006) in favour of their ICN model requires further scrutiny for several reasons:

1. Values estimated for the intermingling of chromatin fibres depend on the threshold chosen for the segmentation of differently painted CTs. Since the choice of the threshold was based on subjective criteria, considerably higher or lower values cannot be excluded.

2. The estimate of 46% (derived from 2.1% x 22 chromosomes) implies the assumption that a given CT is in direct contact with most or all other CTs. This assumption is not consistent with both wet and modelling experiments performed in our laboratories. Our early laser-UV-microbeam experiments showed that chromatin microirradiated during interphase was subsequently only detected on a small subset of mitotic chromosomes (Cremer *et al.*, 1982a; Cremer *et al.*, 1982b). The successful painting of all 24 non-homologous human chromosome types in fibroblast nuclei also demonstrated that any given CT has only a clear minority of all other CTs as immediate neighbours (Bolzer *et al.*, 2005). Using the spherical chromatin domain (SCD) model (Kreth *et al.*, 2004a) our colleague Gregor Kreth estimated an average of 9 neighbours for the CT of a single human chromosome 1 in fibroblast nuclei (G. Kreth, personal communication).

3. The experimental rationale of 24 colour FISH experiments for the multicoloured discrimination of all human chromosomes is based on combinatorial probe labelling schemes with five to seven different fluorochromes (Schrock *et al.*, 1996; Speicher *et al.*, 1996).

4. The successful application of this approach to identify all 24 chromosome types in diploid fibroblast nuclei from human males (Bolzer *et al.*, 2005) should have failed in case of extensive global chromatin intermingling with many giant chromatin loops entering the interior of a given CT from neighbouring CTs.

5. While the intermingling of chromatin loops from neighbouring CTs favours chromosomal rearrangements (Branco and Pombo, 2006), quantitative modelling of chromosomal rearrangements

based on the spherical 1Mbp chromatin domain (SCD) model (a computer model based on the CT-IC model) are in good agreement with published studies on chromosomal rearrangements induced by radiation experiments (Kreth *et al.*, 2007).

A critical reappraisal of current models of nuclear architecture

In contrast to the CT-IC model both the *lattice* model and the ICN model need to explain how cells avoid problems of chromatin fibre entanglements, which generate a hindrance for the separation of chromosomes, when cells enter mitosis. This problem becomes clearly aggravated, if one assumes that numerous giant chromatin loops meander from the surfaces of higher order chromatin structures and form zones of intermingling fibres between such structures, ranging from ~1 Mb chromatin domains to entire CTs. Several reports have provided examples of gene dense regions extending away from their home CT with significantly higher frequency, when these regions become poised to and/or actively involved in transcription. Studied regions include segments from the major histocompatibilty complex locus on HSA 6 (Volpi *et al.*, 2000), the epidermal differentiation complex region on HSA 1 (Williams *et al.*, 2002) and a gene dense region on human 11p15.5 (Mahy *et al.*, 2002a). Mahy *et al.* suggested that local gene density and transcription, rather than the activity of individual genes, influences the organization of CTs. In a review Chubb and Bickmore (2003) argued that transcription decondenses chromosome territories, extruding large loops of chromatin that then collapse back into condensed territories when transcription ceases. Notably, a scheme provided in this review suggests that extruding (30 nm thick?) chromatin loops meander in the vicinity of their home CT. In order to assess the chromatin compaction level of the gene dense region on HSA 11p15.5 we used a BAC contig spanning the entire region (~2.5 Mb) as a FISH probe. We observed that this region occasionally expanded as a rather straight, finger-like chromatin protrusion up to ~2 μm from the surface of the HSA 11 territory. Estimates of the compaction level indicated that the compaction level of these extended structures was one order of magnitued higher than the compaction level of an extended 30 nm chromatin fibre (Albiez *et al.*, 2006).

In addition to the specific example mentioned above, we performed experiments to study chromatin compaction at a more global level. For this purpose, we generated living HeLa cells harboring a few CTs labeled by fluorescent ~1 Mb chromatin domains, while the majority of CTs were unlabeled. Notably, only a fraction of the replication foci was originally pulse-labelled during S-phase with fluorochrome conjugated nucleotides. This fraction yielded the labeled ~1 Mb chromatin domains in CTs observed several cell cycles later in the progeny of such cells. In deconvolved light optical nuclear serial sections we did not find evidence for fluorescent signals clearly above background levels both between individual fluorescent ~1 Mb chromatin domains within a given CT and in nuclear areas between labelled CTs. This evidence argues against a substantial fraction of giant 30 nm thick chromatin loops, which would expand over micrometer distances within or outside their home CT (Albiez *et al.*, 2006).

In a careful electron microscopic study (Visser *et al.*, 2000) labelled cells with BrdU during one S-phase and allowed the cells to divide for several cell cycles they found a few labelled chromatin domains obviously belonging to different CTs. Three major patterns have been observed: 1. complete physical separation of the domain by an interchromatin space; 2. two domains appearing as one continuous chromatin area but exhibiting a sharp separation of label; 3. two domains appearing as one continuous area with a gradual transition of labelling density with chromatin fibres somewhat interspersed between the two domains suggesting the intermingling of chromatin loops.

Structures located within a meshwork of entangled chromatin fibres as proposed by the *lattice* model and the ICN model might become trapped, when chromatin loops condense at the onset of mitosis. Disintegration of such structures during early prophase, as is the case for nuclear speckles (Lamond and Spector, 2003) and PML bodies (Dellaire *et al.*, 2006), provides a way to avoid trapping. The presence of double minute chromosomes (DMs) in certain tumour cells provides an example for a structure, which persists throughout the entire cell cycle (Solovei *et al.*, 2000). In case of the *lattice* model or the ICN model, we would expect that DMs located in the interior of a CT should run a considerable chance of getting trapped within the chromatin fibre meshwork proposed by these models as soon as chromatin fibres retract during

prophase in order to form the much more condensed mitotic chromosomes. Accordingly, we would expect to see at least occasional DMs trapped within chromatids, but this was not the case. DMs were always found between mitotic chromosomes (*data not shown*). Figure 24 shows the topographical relationship between DMs and the painted 3q-arm domain in the nucleus of a human neuroblastoma cell. These DMs contain amplified, transcriptionally active MYCN genes. Confocal serial sections and the 3D-reconstruction of the entire 3q-arm domain show IC channels leading into the interior of the 3q-arm domain, as well as DMs indisputably located not only at the periphery, but also within IC channels penetrating the interior of the arm domain. These observations are compatible with the CT-IC model. Here, invading channels with DMs reflect invaginations of a folded higher order chromatin structure. DMs sitting deeply in these invaginations can be expelled, when the CT becomes stretched at the onset of prophase and forms a prophase chromosome. In the light of the CT-IC model we would predict that DMs like other chromatin domains reveal a perichromatin region, where transcription takes place. The location of DMs with transcriptionally active oncogenes within the IC channel network brings active genes located at the periphery of DMs in close contact with nuclear speckles located within the IC.

In conclusion, the experiments reported by Albiez *et al.* (2006) and the case of DMs hidden in the interior of CTs (Figure 23) make a strong point for the existence of a functionally relevant, contiguous, three-dimensional interchromatin channel network expanding both at certain sites between CTs and in the interior of CTs. At the same time, these experiments clearly show the limits of resolution of present state-of-the-art 3D fluorescence microscopy and 3D image reconstruction based on light optical serial sectioning. The compact masses and glossy surfaces of chromatin domains forming the 3D con-

tiguous network of 3D-reconstructed higher order chromatin domains/bundles have, of course, a hidden structure, which cannot be resolved by this approach.

Evidence that the large majority of chromatin is compacted into ~1 Mb chromatin domains together with electron microscopic evidence that nascent RNA is formed within the perichromatin region of such domains let us suggest that only a small fraction of transcriptionally active chromatin located in the perichromatin region is strongly decondensed at any given timepoint. The full decondensation or ''open'' configuration of entire genes may not be required in preparation for transcription. Instead, a stepwise decondensation, portion by portion, may suffice. According to this hypothesis only a short segment, which is preparing for immediate transcription may be decondensed to the level of DNA wrapped about a nucleosome and finally to the level of a naked piece of DNA that enters the RNA polymerase of the transcription machinery. Other authors have quite different opinions. It remains to be seen whether the contribution of extended 30 nm thick chromatin fiber loops differs strongly in different cell types with highly different states of their overall transcriptional activities.

Although diffusion of individual proteins contributing to the formation of functional transcription and splicing complexes into the compact interior of ~100 kb chromatin domains is likely not restricted, we suggest several reasons why perichromatin fibrils (and larger transcription factories?) may be preferentially built up the perichromatin region at the surface of compact chromatin domains (Fakan, 2004a; Fakan, 2004b). We further consider two functional scenarios:

Scenario a) Possibly partial transcription/splicing complexes may be formed within the IC and – in contrast to single proteins – deterred from diffusion into the compact interior of ~100 kb chromatin domains. This would mean that entire, functional

Figure 24. (page 263) Topographical relationships between double minute chromosomes (red) and thepainted 3q-arm domain (green) observed in the nucleus of a human neuroblastoma cell emphasize interchromatin compartment channels entering the interior of chromosome territories. A) Laser confocal section through a painted 3q-arm-domain (pseudo-coloured green) and double minute chromosomes carrying active MYCN genes (DMs 1-5) (pseudo-coloured red) observed in a nucleus from the human neuroblastoma cell line HD-N-16. DMs denoted by1-5 in A-F are located both at the periphery of the 3q-arm domain and in channel like invaginations of the interchromatin compartment (IC) penetrating into the interior of the arm domain (reprinted from Solovei *et al.*, 2000 with permission). B) These IC invaginations are emphasized in this gray image of the same 3q-arm domain section. Altough DMs 1-5 are not shown here, their numbered sites are indicated to alleviate comparison with A. C – D) Unpublished 3D reconstruction of the entire 3q-domain (green) with all associated DMs (red). This reconstruction was performed from ~200 nm thick light optical serial sections after correction for chromatic shifts and deconvolution. C) top-view D) Side view. The yellow line indicates a perpendicular section through the reconstructed domain. The result is shown in E and F. E) Interior of the reconstructed arm domain seen after removal of the top part. F) View of the reconstructed arm domain from the bottom side. For this purpose the 3D image shown in E was flipped over. Note that DM 1 is still visible from the outside in C and F, although it is clearly located in the interior of the 3q-arm domain. Bars correspond to 1 μm.
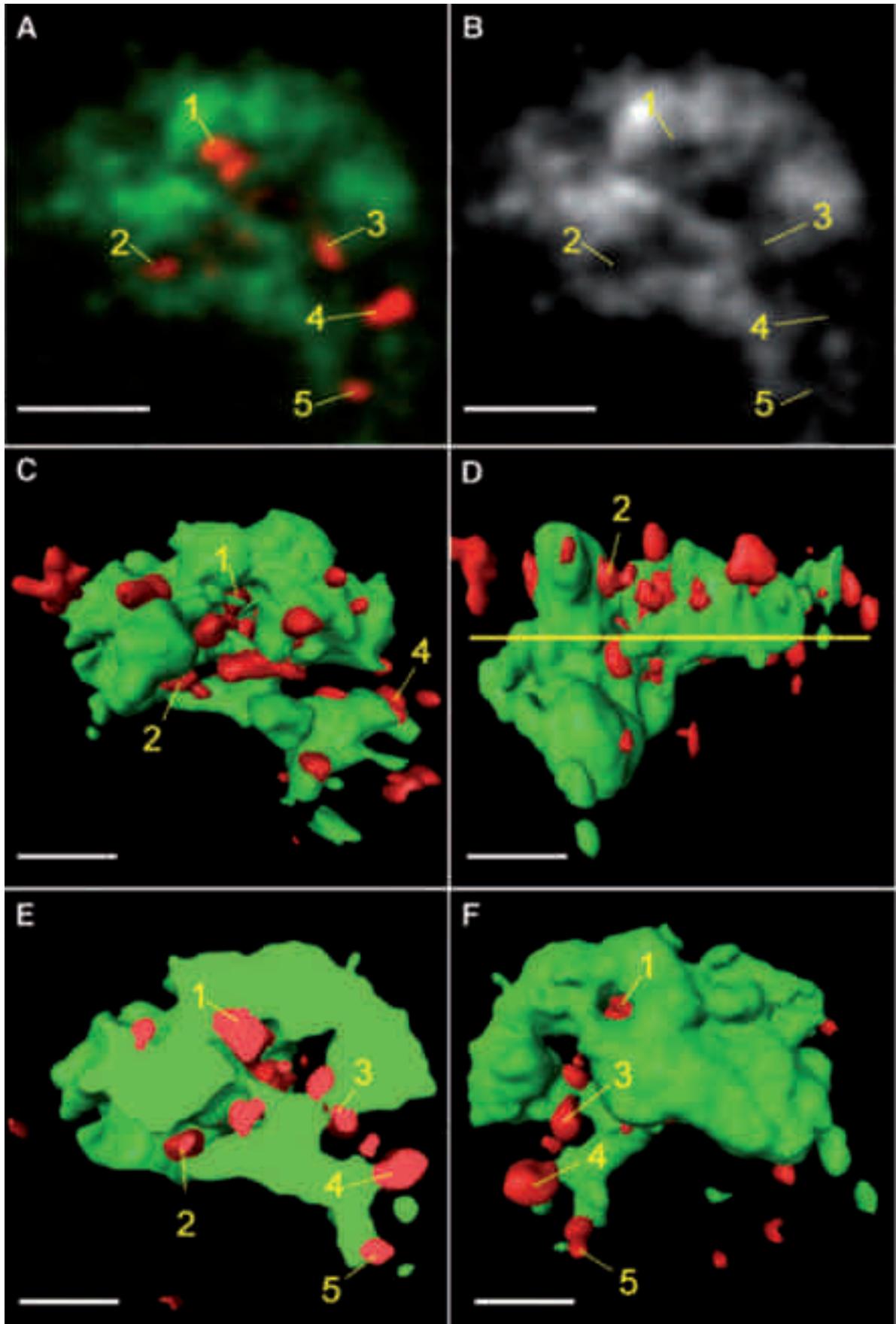
transcription/splicing complexes are built up form modules with constrained diffusion in the perichromatin region.

Scenario b) In this scenario we consider the possibility that entire, functional transcription complexes can be built up step-by-step from individual proteins. In this case it seems essential that promoters are exposed in the perichromatin region, but not in the domain interior. Accordingly, proteins, such as transcription factors, that need to bind directly to the promotor can do so only in the perichromatin region. As a consequence additional protein interactions with DNA bound transcription factors will also necessarily be restricted to the perichromatin region and entire transcription or silencing complexes will emerge in this compartment. In this context it may be advantageous, if transcription and splicing factors are stored in and released from nuclear bodies and nuclear speckles, respectively, located within the IC. The proposed topography between the IC and its boundary (perichromatin) regions implicates that DNA/RNA binding sites for transcription/splicing factors are located close to nuclear bodies and speckles. Along its random diffusion pathway the factor would first pass through the perichromatin region, where it gets a chance of attachment to a specific binding site and only therafter would move into the compact interior of chromatin domains. In case that many silent genes are located in this interior, the chromatin environment of these genes should be compacted in a way that makes the promoter region inaccessible to these factors. Alternatively, promoter regions of all active and silent genes or even entire genes may be exposed towards the finest IC channels expanding at the periphery of ~100 kb chromatin domains. We prefer the view that factors find their specific binding sites by trial and error, while sliding along the DNA is rather restricted to small segments. A factor, which does not find its specific binding sites after realease from a nuclear body/speckle will finally be stored in another body/speckle and released again. The important point of this CT-IC model scenario is a topography, which helps to enhance the probability for a factor to meet a specific binding site simply because specific binding located in the perichromatin region are on average located closer to bodies/speckles compared to a nuclear topography is of randomly intermingling chromatin fibres with all factors contained in the nuclear sap between such fibres.

Whatever the true functional topography *in vivo*, is the above considerations support the view that topography matters with respect to nuclear functions and possibly provides new clues for attempts of quantitative modelling!

Where does the field of nuclear architecture stand today and what needs to be done in the future?

The details and functional implications of higher order chromatin structures *in vivo* are still an unsolved problem. Although terms like "closed" and "open" chromatin are widely used, we lack compelling structural data, which would explain what these terms really mean. We do not know how much of the chromatin at any given time point of the existence of a given cell type *in vivo* is really made up from 10 or 30 nm thick fibres or from large-scale chromatin structures above that level (Belmont *et al.*, 1989) (Albiez *et al.*, 2006). In a most recent review Christopher L. Woodcock argued that ''even with the most sophisticated preparation and imaging techniques, chromatin in nucleo appears quite amorphous … Perhaps the structural principles that have been identified on the basis of work *in vitro* on idealized substrates apply to rather small stretches of chromatin and, in the nucleus, the irregularities derived from variability in linker length, histone modifications, histone variants and bound non-histone proteins dominate.'' (Woodcock, 2006).

Another area of conflicting opinions concerns the existence, size, nuclear topography and functional implications of an interchromatin compartment mostly devoid of DNA. The interchromatin compartment is an indispensable part of the CT-IC model but not of the *lattice* model and the ICN model. One might argue that these three models can be reconciled with each other based on the argument that any space that exists between a meshwork of 10 and 30 nm fibres and other chromatin structures of a still higher organisation reflects could be considered as part of the interchromatin compartment, but this defies the nature of the interchromatin space described by Fakan and others in electron microscopic studies of ultrahin frozen sections (for reviews see (Fakan, 2004a,b).

Current models of the functional nuclear organization are only of a qualitative nature and experiments to test these models have limitations, primarily due to the methodological constraints faced by the field. Due to the necessity of making clear,

experimentally testable predictions, different models tend to overemphasize different features, which may not necessarily exclude each other. Instead they may point to possibilities realized at some nuclear sites and missing at others or during certain stages of the cell cycle or only in certain cycling, postmitotic and terminally differentiated cell types in order to serve the different functional necessities and constraints of different cells. We expect that answers to the unsolved question of higher order chromatin organization of mammalian cell nuclei *in vivo* will vary to some extent with the species and cell type chosen for analysis, yet we do not know the possible range of this variability. How prevalent are chromatin loops expanding in the interior of the IC in different cell types? Branched 10 nm thick core filaments were described in electron microscopic studies as the basic constituent of a nuclear matrix (Nickerson *et al.*, 1997), but it has not been possible to identify the biochemical nature of such filaments to date. While we consider it unlikely that a nuclear matrix is responsible for higher order chromatin organization, there are many unexplored possibilities for local protein aggregations in the IC, which may form a local matrix involved in the organization of a locally defined environment necessary for transcription, splicing etc. (Cremer *et al.*, 1995; Zaidi *et al.*, 2005). Are ~1 Mb chromatin domains seen by fluorescence microscopy simply convolutes of 10 nm and 30 nm thick chromatin fibres or are they more complex structures kept together by presently unknown "linkers" (Albiez *et al.*, 2006). Are giant chromatin loops functionally indispensable for transcription regulation of certain genes, e.g. by enabling "kissing events" between genes located in different bands of a given CT or on different CTs (Lanctot *et al.*, 2006). An old chicken and egg question reflects the relative importance of structural and functional studies in biology. This question, if at all important, has no generally valid answer. Molecular biologists, who remain doubtful, whether the still murky field of nuclear architecture will ever provide unprecedented insights into nuclear functions, should remember that understanding of DNA functions became only possible after Francis Crick, Rosalind Franklin, Jim Watson and Maurice Wilkins had deciphered the structure of DNA in a highly competitive, yet inseparably interdependent effort of ideas and experiments. In this particular case understanding structure was clearly essential for understanding function. While

the discovery of epigenetic mechanisms and the progress in understanding these mechanisms have been extremely rewarding during the last decade, the puzzle of how these and other likely still undiscovered mechanisms interact at all levels and form a cell-type specific epigenome is far from being solved.

Given the still uncomfortable disparity in the field of nuclear architecture between what optimists may call secure knowledge and pessimists unvalidated speculation, attempts of quantitative modelling structure-function relationships of the cell nucleus may appear premature. In fact, only very few attempts have been undertaken so far to develop quantitative models of CTs and their nuclear arrangements (Cremer C., Munkel C. *et al.*, 1996; Cremer T., Kreth G. *et al.*, 2000; Kreth *et al.*, 2004b; Kreth *et al.*, 2004a; Munkel *et al.*, 1999; Münkel and Langowski, 1998; Sachs *et al.*, 1995). We believe that such attempts should be encouraged (and funded!). They are necessary not despite but because of our ignorance of validated principles of structure-function relationships. Conflicting models help to define experiments to support or falsify model predictions (Shopland *et al.*, 2006).

Imaging procedures at all levels of resolution from the Angström scale to the nanometer and micrometer scales are indispensable to understand the space-time structure of molecular machineries and their topography with regard to higher order chromatin domains/bundles and the interchromatin compartment. Further insights into the functional organization of molecular machineries for transcription, splicing, DNA-replication and repair beyond the biochemical description of more and more proteins, which contribute to a given machinery, require ever advanced methods of crystallography and cryo-electron microscopy. Advancements of light microscopic resolution beyond the Abbe limit will help to bridge the present gap of resolution between light and electron microscopic approaches and for the first time allow studies of the nuclear topography of such machineries in the nucleus of living cells at unprecedented resolution. There is no single method, which would be superior in every aspect to other methods. What matters is the combination of all possible approaches in most suitable ways to pursue the final goal to understand in which way nuclear architecture matters with respect to nuclear functions. It seems obvious that understanding the structure-function relationships

of a biological system – in our case the cell nucleus – can not be achieved by studying this system only at one level. The real task is to understand a given biological system in toto including the impact of its environment. If we wish to understand the cell nucleus in this context, it must be studied at the molecular level, the chromatin level, the chromosome level, the nuclear architecture level and beyond, i.e. the nucleus in the context of its environment the cytoplasm, the cell membrane and other cells, which affect the function of this nucleus by signalling events. Unfortunately, the new buzzword systems biology has often been restricted to molecular systems biology, While high throughput analyses the gene expression status and the proteom of cells have been established as a highly welcome and indispensable part of cell and developmental biology, it is also obvious that the meaningful handling of the enormous data sets collected in the fields of genomics, proteomics and so forth is a huge task. Despite the beauty of algorithms, which yield impressive schemes of interconnected hubs of ever increasing complexity, one must be aware that it is still difficult to decide, which of the newly generated hypotheses strong and which are rather weak and possibly misleading. Most of the real work of proving functional connections triggered by such hypotheses still needs to be done. At this state a single-minded focus on high throughput methods is not helpful. Structural biology at the level of single cells from molecules to molecular machineries, to chromatin and chromosomes, to the nuclear architecture and the cell and other surrounding cells at large is indispensable, if we focus on the long term goal of really understanding as best as possible how a living systems works. At the molecular level the methods required for such work include cristallography and cryo-electron microscopy of individual proteins and protein complexes. While work at this level has been generally accepted as a necessary part of functional studies, quantitative structural studies carried out at higher levels of organization have often been negatively labelled as purely descriptive. Notwithstanding the obvious importance of hypothesis driven experiments, the valid quantitative description of a given system of nature is sometimes the only way to proceed in a particular field. Astronomers can image distant galaxies, they can generate hypotheses, but studies of the structure of the universe at large will forever remain the domain of pure and wonderful imaging, while ideas to manipulate the physical

world will be restricted to the small scale of our mesocosmos. Gladfully, the little universe of the cell nucleus is amenable to experimental interference, yet the interpretation of experimental manipulation requires secure, quantitative knowledge of how nuclear phenotypes differ in a variety of cell types and species. In our view the generation of secure, descriptive knowledge is the most important task, which has still to be solved. Only on such a secure basis can be expect that efforts to elucidate mechanisms, which link higher order chromatin arrangements and other aspects of the nuclear architecture with nuclear functions. To be sure, efforts to understand a possible impact of nuclear structure on nuclear function require model systems as well in due course, which allow the experimental manipulation of certain features of the nuclear architecture either by directly manipulating the location of chromatin or by manipulating genes, which affect nuclear architecture. The emerging field of laminopathies as a consequence of mutations of the gene for lamin provides a case in point (Broers *et al.*, 2006; Maraldi *et al.*, 2006; Mattout *et al.*, 2006; Parnaik and Manju, 2006). These mutations affect the specific attachment of chromatin to the lamina with most serious consequences, as shown for example by the development of the Hutchinson-Gilford syndrome with its symptoms of premature aging (Eriksson *et al.*, 2003). The pathological nuclear phenotype can be induced, when a cell line is transfected with the mutated gene (Goldman *et al.*, 2004). It would be most desirable to pinpoint other genes with mutations that affect the nuclear phenotype. Knock-in and knock-out or knock-down experiments of such genes in model cell cultures or animals would then provide a chance to understand the implications of nuclear architecture for cell functioning at a mechanistic level. The interpretation of such experiments, however, may turn out to be much less straightforward than one may hope, since genes act within complex genetic and epigenetic networks. The set of complex problems, many of them likely not even clearly formulated to date, which must be solved for a true understanding of the functional implications of cell type specific epigenomes and the interplay of epigenetic mechanisms at all levels from DNA methylation to nuclear architecture at large, likely provides obstacles of a magnitude as difficult to overcome as the obstacles that prevented the understanding of genomes and genetic mechanisms during the first half of the 20th century.

Acknowledgements

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