Organization of chromosomes in the interphase cell nucleus
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Chromosomes and the interphase cell nucleus
- general introduction -

The genome in the nucleus of every cell is well organized. The enormous amount of DNA that carries the genetic information asks for a specific spatial organization. For example, the nucleus of a human cell contains a string of 2 meters of DNA, which corresponds with 20 km of thread in a tennis ball. To allow correct division of this DNA over the daughter cells, the nuclear genome is divided over a number of chromosomes.

During interphase, the nucleus is compartmentalized in various domains with specific functional and structural characteristics. A whole range of mechanisms ensure that the chromosomes remain intact, that they are duplicated before cells divide and that the correct genes are transcribed at the right time. Although many of the underlying mechanisms are well understood at the molecular level, questions remain unanswered as how these processes take place in the context of nuclear structure. One of the unanswered questions is whether the organization of chromosomes in interphase is functionally related to the organization of nuclear processes. This question has been addressed in the present thesis.

Chromosomes in metaphase

Chromosomes are known since the 19th century. It was then observed that nuclei compacted into rod-like structures during cell division (Fig. 1). In 1888 these structures were named ‘chromosomes’, after the Greek words chromo (color) and soma (body), because they stained particularly well with the dyes that were used for microscopic preparations. After cell division, when chromosomes have been equally divided over the daughter cells, chromosomes decondense and cannot be recognized anymore as distinct structures (Fig. 1). Therefore, most of our understanding of chromosomes
Fig. 1 Scheme of cell cycle. During cell division, chromosomes condense into rod-like structures (a-d). These are pulled apart and separated equally over the two daughter cells (M, mitosis) (d-f). Then, chromosomes decondense and are not recognizable as individual structures anymore (G1) (f). Before the next cell division, cells replicate their DNA in the S (synthesis) -phase and prepare for actual division in G2 (gap)-phase (a). Cell division (M) may last approximately 30 min. G1, S and G2-phase, together called interphase, may last several hours in proliferating cells or days to years in resting cells. Images a-f from CD-ROM: "Understanding Mitosis and Meiosis; an interactive educational tool" (courtesy of Oof Oud and Geoff Rickards).

Chromosomes have a well defined structure during metaphase (Fig. 2). All chromosomes are duplicated before mitosis and these identical copies, chromatids, are aligned next to each other in metaphase. A small constriction identifies the centromere where the mitotic spindle, that is temporarily made from microtubuli, is attached to specialized chromatin to pull the two chromatids to each side of the dividing nucleus. The telomeres at the ends of chromosomes are specialized structures as well that maintain chromosome integrity. A subset of chromosomes shows antennae, which are part of the nucleoli in interphase. The length of these antennae may differ between individuals. Other features are recognized when specific dyes are used. These dyes create reproducible patterns of light and dark stained bands on the metaphase chromosomes. The most commonly used banding techniques are G-banding (using the dye Giemsa) and R-banding (Reverse to G) resulting in a complementary pattern. It is not completely understood what causes the differences in staining, but specific characteristics have been assigned to the chromatin in these bands. R-bands have a high gene density, high levels of acetylation of histone proteins (which are associated with active genes), high density of G-C base pairs, they replicate early and are relatively often involved in chromatid exchanges. In contrast, G-bands have a low gene density, low levels of acetylation, high density of A-T base pairs, replicate later and are less involved in chromatid exchanges (reviewed in Holmquist, 1992; Craig and Bickmore, 1993). These banding patterns are very char-
characteristic and each individual chromosome can be identified in metaphase spreads based on their size and banding patterns (Fig. 2).

**Chromatin organization**

After mitosis, the chromatids are separated and decondense in the newly formed nuclei. The chromosomes, then, cannot be recognized anymore as individual structures. Only little is known about their organization (Fig. 3). Their DNA is never present as simple linear chains of nucleotides which encode the genetic information. At the basal level of organization, DNA consists of two complementary strands of desoxynucleotides that form a double helix in which the nucleotides adenosine (A) are opposed to thymidine (T) and guanine (G) to cytosine (C) (Fig. 3). This helix is wound around a complex of histone proteins and forms arrays of nucleosomes separated by short stretches of so-called linker DNA. Recent studies of isolated nucleosome arrays showed that chromatin folds into an irregular zigzag and forms fibers of 30 nm diameter (Fig. 3) (reviewed in Woodcock and Horowitz, 1995). Folding depends on several factors, such as length of linker DNA between nucleosomes, presence or absence of linker histones that tie linker DNA segments, and local ion concentrations (Bednar et
Fig. 3 Several levels of chromatin organization. a) Genetic information is encoded in long sequences of nucleotides A, T, C and G. These form together with a complementary strand a DNA double helix. Distance between sequential nucleotides is 0.34 nm (I). The helix is wound 1.7 times around a complex of histone proteins, forming arrays of nucleosomes (for scale: diameter of nucleosome is 10 nm) (II; interpretation after Woodcock et al. 1995). Arrays of nucleosomes fold into an irregular 3D zig-zag, forming a chromatin fiber with a diameter of approximately 30 nm (III; interpretation after Bednar et al. 1998). b) Higher levels of chromatin organization are still unclear. It may be that the 30-nm fiber folds into so-called chromonema with diameters of approximately 100 nm, which again may fold into higher order chromatin fibers (interpretation after Belmont, 1997). c) At the nuclear level, chromatin of a single chromosome is organized into a chromosome territory (bright areas in nuclei) and during cell division condensed into rod-like metaphase chromosomes (chromosome 8 is painted on human nuclei and metaphase spread).

Removal of linker histones results in less regular folding and lower compaction of DNA (Bednar et al., 1998). DNA-dependent processes, such as transcription and replication, are believed to utilize low compacted chromatin. This concept is consistent with observations that linker histones may inhibit transcription (O’Neill et al., 1995; Wolffe et al., 1997) and indicates that chromatin folding in the nucleus is regulated in a functional manner. In vivo, very few fibers of 30 nm are observed in nuclei. Most chromatin is more compacted, which suggests a higher order of chromatin organization. Fiber-like structures of various diameters have been found in interphase nuclei (e.g., Sedat and Manuelidis, 1978; Belmont and Bruce, 1994; Robinett et al., 1996), but the clearest indication of a functional organization of chromatin is that individual chromosomes form local territories (reviewed in Cremer et al., 1993). Various models have proposed how chromatin is organized in interphase nuclei and how it condenses into metaphase chromosomes (e.g., Marsden and Laemmli, 1979; Boy de la Tour and...
general introduction

Laemmli, 1988; Manuelidis, 1990; Belmont, 1997) but the exact folding is still not understood properly.

Chromosomes in interphase

Not long after chromosomes had been discovered, Rabl (1885) proposed that they remain positioned in interphase nuclei as they are separated during mitosis. He deduced by studying dividing cells that the centromeres, at which site the chromatids are pulled apart by the mitotic spindle, are localized at one site of the new nucleus and that the telomeres, which lag behind during the separation process, are located at the opposite side. This is now called a Rabl-configuration and has been observed in various cell types. The concept that chromosomes remain as structural entities in specific territories during interphase was proposed also by Strasburger (1905) and Boveri (1909) in those early days.

In the decades that followed, these ideas were neglected because the existence of chromosome territories could not be proven. Furthermore, the general excitement about the discovery of the molecular structure of DNA by Watson and Crick in 1954 and its direct implications for DNA to carry and reproduce genetic information correctly, directed the general interest to molecular mechanisms of transcription and replication, rather than structural issues of chromatin organization. As electron microscopic (EM) studies failed to detect individual chromosomes, the concept of a territorial organization was abandoned in the sixties and early seventies. It was then generally assumed that chromosomes consisted of fibers with diameters of 10 and 30 nm which had many attachment sites with the nuclear envelope and are intermingled in the nucleoplasm (Comings, 1968). Chromatin fibers were thought to be intermingled in the nucleus as spaghetti in a bowl. This metaphor has been tested in practice. H. G. Davies mixed spaghetti in hot gelatin in a smooth bowl, examined the surface after cooling and made sections. He concluded that the resulting images were similar to those made of sections through cell nuclei (Davies et al., 1974).

However, the principle of territorial organization of chromosomes has revived. Experiments showed that when small regions of the interphase nucleus are damaged by localized irradiation, DNA repair is detected on a few metaphase chromosomes only (Cremer et al., 1982). These findings indicate that chromosomes are not distributed throughout the interphase nucleus but remain in territories. Interphase chromosomes were visualized for the first time in hybrid cell lines, where one human chromosome was inserted in the nucleus of a mouse cell. In situ hybridization with total human DNA demonstrated that this single chromosome occupied a small region in the nucleus only (Manuelidis, 1985; Schardin et al., 1985). Shortly after that, probes were made of DNA that was derived from a single chromosome. By suppressing the repetitive sequences that are found on all chromosomes, this chromosome could be specifically labeled in interphase nuclei and was found to form a territory (Cremer et al., 1988; Lichter et al., 1988; Pinkel et al., 1988; Leitch et al., 1990; Heslop Harrison, 1990).
This technique has become generally known as ‘chromosome painting’, a suitable name, as each chromosome in a metaphase preparation now can be delineated in a different color (Schröck et al., 1996; Speicher et al., 1996).

**Nuclear compartmentalization**

It was then established that chromosomes occupy distinct territories. Furthermore, it was proven that nuclear processes are not randomly distributed over the nucleus. A good example is DNA replication. The machinery that replicates DNA does so with a speed of 100 nucleotides per second in human cells. If DNA synthesis would start at one end of a chromosome and replicate the nucleotides one by one in a single row, it would take more than a month before a large chromosome is replicated. Therefore, DNA replication is initiated at multiple sites on chromosomes of eukaryotic cells (mammalian cells have approximately 100 000 origins of replication). Replication is activated simultaneously in clusters of 20-80 adjacent origins of replication. The DNA within such a replicon cluster is replicated in less than one hour (Edenberg and Huberman, 1975; Hand, 1978). Replicon clusters fire in a highly reproducible temporal and spatial order to replicate all DNA in a mammalian nucleus in 5-12 h. In the nucleus, three (Nakayasu and Berezney, 1989) to five (Van Dierendonck et al., 1989) spatial distribution patterns of replication sites can be recognized during S-phase progression: replication starts in several hundreds of small foci distributed all over the nucleus, later in S-phase chromatin located near the nuclear envelope and around the nucleolus replicate, while last replicating chromatin is clustered in a few large domains in the nuclear interior (Fox et al., 1991; Manders et al., 1992) (Fig. 4). Interestingly, DNA that is replicated early in S-phase will again replicate early in daughter cells, and DNA that is replicated late always replicates late, cell generation after cell generation (Jackson and Pombo, 1998; Ma et al., 1998). This was also noticed in metaphase chromosomes in which DNA that is labeled during early S-phase appears as a banding pattern that corresponds to R-bands, and DNA that is labeled during late S-phase corresponds to G-bands (Drouin et al., 1994; Ferreira et al., 1997; Zink et al., 1999). The reproducible distribution of early and late replicating chromatin in distinct domains over several cell genera-

**Fig. 4.** DNA replication occurs in foci that are distributed over the interphase nucleus according to characteristic patterns. a: replication in early S-phase; b: replication in late S-phase, in human skin fibroblast. Bar 2 μm.
tions indicates that these domains form stable units in the cell nucleus.

Other nuclear processes are compartmentalized as well. Transcription occurs in several hundreds of foci distributed over the nucleus in a way similar to early replication sites (Jackson et al., 1993; Wansink et al., 1993). Although RNA transcription can take place during replication of a replicon (McKnight et al., 1978; Liu et al., 1993), it is not clear whether transcription and replication indeed do occur simultaneously in the same foci (Hassan et al., 1994) or not (Wansink et al., 1994; Wei et al., 1998). Production and processing of ribosomal RNA take place in a specialized compartment. The chromosomal loci that contain ribosomal genes form nucleoli together with RNA, proteins and other factors. Nucleoli are one of the best described nuclear compartments where structure and function have been shown to be closely related (Scheer and Benavente, 1990).

The genome as it appears in electron micrographs of interphase nuclei has been categorized in two forms: compact heterochromatin, which is localized predominantly in the nuclear periphery and around nucleoli in clumps in the interior, and less densely packed euchromatin in the remainder of the nucleus. Generally, it is an oversimplified concept that euchromatin represents actively transcribed chromatin and that heterochromatin is inactive with respect to transcription. Heterochromatin can be subdivided in constitutive heterochromatin, which is always heterochromatic, and facultative heterochromatin, which switches between euchromatin and heterochromatin in a controlled manner (for review, see Spector, 1993).

Recent studies have shown that heterochromatin compartments may possess transcription inactivating capacities (reviewed in Cockell and Gasser, 1999). In yeast, a heterochromatic compartment is formed by a cluster of telomeres near the nuclear periphery. In one study (Andrulis et al., 1998), a DNA construct that can be artificially attached to the nuclear envelope, was introduced into the yeast genome. This gene was actively transcribed when it was located in the nuclear interior, but inactivated when it was targeted to the nuclear envelope in the heterochromatic compartment. Another study demonstrated that in maturing lymphocytes, a subset of silenced genes is located near centromeric heterochromatin, associated with the protein Ikaros. After differentiation of the cells, this subset of genes is released from Ikaros and is actively transcribed in the nuclear interior, while other genes become associated with Ikaros and are targeted to centromeric heterochromatin (Brown et al., 1997). It is thus clear that both the genome and nuclear processes are compartmentalized in functional domains in the interphase nucleus (for reviews, see e.g., Spector, 1993; Van Driel et al., 1995; Strouboulis and Wolffe, 1996; Lamond and Earnshaw, 1998; Schul et al., 1998; Bridger and Bickmore, 1998; Cockell and Gasser, 1999).

**Implications of territorial organization of chromosomes**

The next question is how these nuclear compartments relate to chromosome territories. Several studies showed that mRNA transcribed from DNA of an integrated virus
(Zirbel et al., 1993) and the few genes studied so far (Cremer et al., 1993; Kurz et al., 1996; Park and DeBoni, 1998) are located preferentially near the surface of chromosome territories. Speckles which are rich in proteins involved in mRNA splicing are also preferentially located outside chromosome territories (Zirbel et al., 1993). These findings lead to the hypothesis that there is a space in between chromosome territories which enables transport of factors and assembly of enzyme complexes. This space facilitates nuclear processes, such as transcription, replication and DNA repair to take place preferentially at the surface of chromosome territories (Cremer et al., 1993; Zirbel et al., 1993). The interchromatin domain (ICD) space model was later extended with channels penetrating into chromosome territories to facilitate processes at the surface of chromosomal subdomains (Cremer et al., 1995). The ICD model suggests that chromatin is organized inside chromosome territories in a function-dependent manner.

Territorial organization of chromosomes implies restrictions with respect to probability of chromatin interactions. There are, however, indications that at least some genes interact with one another during interphase (LaSalle and Lalande, 1996; Wu and Morris, 1999). Also during (mis-)repair of DNA double strand breaks, chromatin of one chromosome may interact with a double strand break in another chromosome resulting in chromosome translocation (for review, see Savage, 1996). Thus, either this chromatin is already closely associated when damage is induced, or broken chromosomal ends are transported and become connected during repair.

To elucidate mechanisms that are involved in these processes, it is essential that chromosomal structure is studied besides molecular backgrounds of the mechanisms. The following type of questions have to be addressed in order to understand nuclear processes, such as replication, transcription and repair. Are chromosomes indeed organized in such a way that nuclear processes take place preferentially in specific compartments? And, does chromatin of different chromosomes intermingle (e.g., similar to the spaghetti model) or do chromosomes form mutually exclusive units?

### Techniques applied to study chromosomal organization

To study these questions, one has to detect first chromosomes in interphase nuclei. The most common way to label individual chromosomes is by chromosome painting, as mentioned above. This technique is based on the high affinity of single stranded DNA for its complementary sequences, which causes them to bind specifically. Probes that are used for the detection of whole chromosomes are generally synthesized by isolating specific chromosomes, e.g. by fluorescence flow activated sorting, which are then fragmented, multiplied, and tagged with a label (see e.g., Stap et al., 1998). However, not all sequences within a specific chromosome are unique to that single chromosome. Binding of probe DNA to repetitive sequences in other chromosomes reduces specificity of painting of chromosome territories. This crosshybridization can be efficiently suppressed by allowing a surplus of unlabeled repetitive sequences to pre-anneal (hybridize to the probe) before the probe is applied to nuclei (Cremer et al., 1988;
Lichter et al., 1988; Pinkel et al., 1988). Hence, the official name of this procedure is chromosomal in situ suppression hybridization (CISSH).

To investigate where nuclear processes take place with respect to chromosome territories, we combined this technique with the labeling of replicating DNA with halogenated thymidine analogues (Aten et al., 1992; 1994). Replication double labeling allows detailed analysis of spatial and temporal progression of the replication process (Aten et al., 1992; Manders et al., 1992; 1996; Ma et al., 1998). For our studies, we further exploited the use of halogenate thymidine analogues. We labeled not only sites of replication but also chromatin domains with particular characteristics (e.g. early or late replication) and entire chromosomes without applying CISSH. Furthermore, the double labeling allowed us to determine the cell cycle phase of individual cells selected for imaging within an asynchronous cell population. Since DNA replication double labeling is a major technique used in this thesis, the principles of this method will be shortly addressed and an overview of its multiple applications in this thesis is given.

**Principles of replication labeling and its detection**

Halogenated thymidine analogues, such as bromo-, iodo-, and chlorodeoxyuridine (BrdU, IdU, and CldU, respectively) are accepted by endogenous replication enzymes as substrates. They diffuse through the cell membrane and can therefore simply be added to the culture medium of cells to be incorporated into newly synthesized DNA in vivo (Van Driel et al., 1998). At low concentrations (≤ 10 µM) these analogues are virtually nontoxic (Aten et al., 1994; Van Driel et al., 1998). They may, however, induce fragile sites (Stone and Stephens, 1993) which result in sister chromatid exchanges after prolonged culture periods (chapter 4). Unincorporated analogues are washed away by several changes of medium. Since a specific set of antibodies discriminates between IdU and CldU (all recognize BrdU), chromatin replicated during different periods can be detected in one and the same nucleus (Aten et al., 1992). Replicating chromatin is labeled first by IdU, then the unincorporated analogues are washed away, and after a specific time interval (chase period) replicating chromatin is labeled by CldU. The analogues are then detected by immunocytochemistry after chemical fixation of the cells (Gratzner, 1982; Aten et al., 1992). Double stranded DNA is denatured to allow the primary antibodies to recognize their epitopes. Secondary antibodies that are conjugated with either fluorochromes for detection by fluorescence microscopy or colloidal gold particles for detection by EM are then used to specifically localize the thymidine analogues in nuclei.

By varying labeling and chase periods, we developed a series of protocols to label several phenomena with halogenated thymidine analogues in interphase nuclei (Stap et al., in preparation).
1. Analysis of cell cycle phase

When cells are labeled with IdU and after a chase period with CldU, four classes of cells can be recognized: 1) cells labeled with IdU only: these cells were in S-phase at the moment of IdU exposure, but had finished replication when CldU was added; 2) cells labeled with CldU only: these cells were not in S-phase during IdU exposure, but had entered S-phase during the chase period; 3) cells labeled both with IdU and CldU: these cells were in S-phase during IdU exposure and CldU exposure; 4) unlabeled cells: these cells were not in S-phase during either IdU or CldU exposure (Van Driel et al., 1998). When the duration of the cell cycle is known, chase periods can be chosen to allow selection on the basis of the presence or absence of incorporated analogues of cells that were chemically fixed in a specific phase of the cell cycle. Examples are given in Table I. We applied this method to compare distribution patterns of replication sites over chromosome territories in early and late S-phase (chapter 2).

Table 1. Analysis of cell cycle phase

<table>
<thead>
<tr>
<th>observation</th>
<th>conclusion</th>
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<tr>
<td>IdU incorporated</td>
<td>CldU incorporated</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
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<tr>
<td>No</td>
<td>Yes</td>
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<td>Yes</td>
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Examples of cell cycle analysis using V79 Chinese hamster cells. The cell cycle phase of a particular cell as well as the cell cycle phases in which its chromatin was labeled can be deduced from the presence or absence of incorporated thymidine analogues. Flow cytometry showed that V79 cells have the following cell cycle kinetics: G1 lasts 2h, S lasts 5h, G2/M lasts 3h. In this example, cells were labeled briefly with IdU, chased for 1-4 h, labeled with CldU and fixed immediately afterwards.

2. Sites of replication

Sites of replication are marked by a short pulse label directly before chemical fixation of cells. Strictly speaking, one does not label sites where replication actually takes place, but DNA that was synthesized during the last few minutes. However, after this relatively short period between DNA synthesis and fixation, labeled chromatin is found at sites of replication, especially at the LM resolution (Manders et al., 1992; 1996). We applied this labeling method to analyze spatial distribution patterns of replication sites within chromosome territories (chapter 2).
3. Chromosomal subdomains

Early and late replicating chromatin have different characteristics. Early replicating chromatin generally contains active genes and late replicating chromatin consists of heterochromatin (Goldman et al., 1984). By applying replication labels with a chase period of approximately half the duration of the S-phase in between, these types of chromatin are labeled individually. Cells that contain both labels had thus incorporated IdU in the first half of S-phase and CldU in the second half (Table I). Only this subset of cells is analyzed. We developed this approach in our studies to directly compare distribution patterns of early replicating chromatin and late replicating chromatin over chromosome territories (chapter 2). Similar approaches have recently been used by Ferreira et al. (1997) and Zink et al. (1999).

4. Chromosomes

The semi-conservative character of DNA replication, in which one DNA strand remains intact and serves as a template for a newly synthesized strand, allows labeling of a subset of chromosomes by thymidine analogues. When thymidine analogues are present during the entire S-phase, all newly synthesized DNA is labeled and present in one strand of the DNA double helix (Fig. 5). These DNA helixes coil and fold into chromatids. In metaphase, all chromosomes contain two completely and uniformly labeled sister chromatids. In the next S-phase, in the absence of thymidine analogues, a labeled DNA strand serves as template for a new unlabeled strand and remains itself labeled. The original unlabeled strand serves as template for the other copy, and forms together with the unlabeled newly synthesized strand an unlabeled DNA double helix. In the following metaphase, all chromosomes contain one labeled chromatid and one unlabeled chromatid. During subsequent cell cycles, the labeled chromatids are diluted over generations of daughter cells so that only a few chromosomes or no chromosomes at all are labeled per nucleus (Fig. 5).

When cells are cultured during one S-phase in the presence of IdU, during the next S-phase in the presence of CldU, and during a third S-phase in the absence of thymidine analogues, chromatids are labeled with either IdU, or CldU or are not labeled at all (see e.g., chapter 3, Figs. 1 and 5). After mitosis, in the G1 phase of the fourth cell cycle, these chromatids correspond to single chromosomes that are either labeled with IdU, with CldU or unlabeled.

During the replication process sister chromatid exchanges occur. This leads to an abrupt change in label in a chromatid. These sister chromatid exchanges are visible in metaphase and result in a harlequin staining pattern. In these cases, chromosomes are not labeled completely, but domains within chromosomes are labeled exclusively with either IdU, or CldU or are unlabeled. These domains therefore provided information on the internal organization of chromosomes as well. We applied the single labeling approach in our EM studies (chapter 5) and developed the double labeling approach to determine whether chromosome territories intermingle, as is described in chapter 3.
Fig. 5. Labeling of chromosomes by incorporation of thymidine analogues. When thymidine analogue BrdU (black) is incorporated in newly synthesized DNA during the first S-phase (S), one strand of the DNA double helix is labeled. During metaphase (M), chromosomes appear entirely labeled as the two strands of the helix cannot be resolved. When during the second cell cycle BrdU is not present, newly synthesized DNA is unlabeled (gray), while the initially labeled strands remain labeled. Metaphase chromosomes now contain one labeled and one unlabeled chromatid. These are separated over the two daughter cells so that, on average, in G1-nuclei in the third cell cycle, half of the chromosomes are labeled and half of the chromosomes are unlabeled. During sequential cycles, labeled chromatids are diluted in a pool of unlabeled chromatids. After several cell cycles in which the labeled chromatids are randomly distributed over daughter cells, individual cells contain none or one to a few labeled chromosomes per nucleus.

Recently, it was demonstrated that chromosomes labeled by directly fluorescence-marked thymidine analogues according to the same principles, can be followed in living cells (Zink et al., 1998; Manders et al., 1999). Unfortunately, these analogues have to be introduced mechanically to the cells and label only part of the chromosomes. Even so, these procedures are a break-through in the analysis of dynamics of chromosomal organization.

**Replication labeling versus painting of chromosomes**

Chromosomes can thus be labeled by CISSH or by replication labeling. What are the differences in results obtained by these two techniques?

Chromosome painting is extremely useful when specific individual chromosomes have to be analyzed. However, chromosome painting is not the most suitable technique to analyze surface areas of territories in general. Labeling of chromosomes with CISSH and suppression of repetitive sequences sometimes lead to incomplete staining of the painted chromosome. Incomplete staining can be observed in metaphase chromosomes as unstained or weakly stained bands. On the other hand, complete suppression of re-
petitive sequences can never be obtained. Therefore, some crosshybridization signal is usually produced elsewhere in the nucleus. These problems limit the accuracy of assignment of labeled chromatin to a territory. In particular, when more than one chromosome is labeled, crosshybridization occurs more frequently. When only two chromosomes are labeled, for example one in red and one in green, one has to analyze many cells in a preparation because only few cells are found in which these chromosome territories are adjacent.

To address the question whether chromatin of different chromosomes intermingle in contact regions of territories (chapter 3), we labeled chromosomes with IdU or with CldU to study boundary areas between two adjacent differently labeled chromosome territories. This approach has the advantage that many of the chromosomes in a cell are labeled and that these chromosomes are labeled uniformly. A disadvantage of the method is that sister chromatid exchanges may cause that only a segment of a chromosome is labeled. Evidently, one cannot assign a labeled domain in interphase to a specific chromosome.

Another aspect of fluorescent in situ hybridization (FISH) is that carefully performed FISH preserves the nuclear morphology well enough for most light microscopical studies but fine details may be blurred (Robinett et al., 1996). Labeling of chromosomes by thymidine analogues is performed in vivo, and their detection involves less harsh preparation conditions. Furthermore, replication-labeled chromosomes can also be detected at the ultrastructural level in well preserved nuclei (chapter 5), or in vivo (Zink et al., 1998; Manders et al., 1999).

In conclusion, when specific chromosomes have to be analyzed, chromosome painting by FISH should be used. Otherwise, replication-labeling of chromosomes can be considered as an alternative.

Microscopy

Labeled nuclei were imaged by confocal laser scanning microscopy (CLSM) and electron microscopy (EM). A CLSM is a fluorescence light microscope with a narrow depth of field. This enables the reconstruction of a 3D image of a cell by recording images as optical sections at different levels in that cell. This is achieved by using a laser as light source that is focused in a single point in the cell. Light emitted by fluorochromes that are located in this focal point is digitally recorded. Light emitted by fluorochromes located outside the focal point is blocked by a pinhole in front of the detector. By moving the focus through the cell, point by point images of the complete cell are recorded and saved in a digital image file (Brakenhoff et al., 1989).

The principles of transmission EM are similar to those of light microscopy, but electrons are used instead of light. The beam of electrons is focused in the specimen by magnetic coils instead of glass lenses. Heavy metals locally absorb or scatter electrons, removing them from the beam as it passes the specimen. An image is formed on a plate that lights up when it is hit by electrons. To record images, a photographic plate
is placed in the electron beam. For immunocytochemical purposes, antibodies are used to which electron dense colloidal gold spheres are attached. These gold particles with a diameter between 5 and 20 nm appear as dark spots in the images. Double labeling is possible by using gold spheres with different diameters, e.g., 6 nm and 15 nm (chapter 4).

EM provides high resolution, but is an elaborate technique. 3D information is even harder to obtain (one has to make serial sections and trace and image the same nucleus through all sections). CLSM records more cells faster and 3D data are relatively easily obtained, but the resolution is limited. Therefore, these techniques complement each other well. However, data obtained by CLSM cannot easily be compared with data obtained by EM. The signal observed in a single confocal optical section represents label present within a layer with a depth of approximately 750 nm (axial resolution), whereas in EM only the surface of a section is labeled by the antibodies. Spatial relationships over larger distances (especially in 3D) are difficult to recognize with EM and small or rarely occurring structures will be present in only a small fraction of the sections. In conclusion, CLSM provides information on the overall organization of the nucleus, whereas EM provides detailed information. These differences in approach may both hamper comparison of structures as observed by CLSM and EM and provide complementary information.

In our case, CLSM and EM studies supplemented each other well. The overall 3D organization was first studied by CLSM and we then focused on more details using EM. To facilitate the direct comparison between the results, we imaged chromosome territories in one and the same nucleus first by CLSM and then by EM. The major advantage of combined CLSM and EM studies is that they provide a link between two approaches, that each yield their own type of information. The combination of techniques can therefore quickly lead to new insights in nuclear structure.

Outline of the thesis

The studies described in this thesis focus on the organization of chromosomes in interphase. In order to establish whether the surface of chromosome territories is a preferred site for nuclear processes (Cremer et al., 1993, 1995; Zirbel et al., 1993), we labeled replicating chromatin in the nucleus in combination with painting of specific chromosomes. We show that DNA replication is not limited to the chromosome surface, but takes place throughout chromosome territories. In addition, we studied distribution patterns of early replicating (gene-rich) and late replicating (gene-poor) chromatin in chromosome territories. Both types of chromatin were found throughout chromosome territories, except for the inactivated X-chromosome. In that chromosome, early replicating chromatin appeared to be preferentially located near the territorial surface. Details are provided in chapter 2.

In chapter 3, we addressed the question whether chromosome territories are separate entities that have a surface or that chromosomes intermingle at their surface re-
regions. Chromosome painting is not the most suitable method to investigate chromosomal borders due to a number of technical problems as we explained above. Therefore, we developed a method based on the incorporation of halogenated nucleotides in DNA during replication and analyzed border areas between sets of differently stained chromosomes in one nucleus. Results obtained by CLSM show that chromosomes are individual entities that do not intermingle. Chromatin fibers extended sometimes from the territories and embedded fibers extending from other chromosomes. Separate segments of chromosomes are remarkably distinct as well and formed non-intermingling domains.

To analyze chromosomal organization in more detail, we developed a method to detect thymidine analogues in well-preserved nuclei at the ultrastructural level using EM. The results show that IdU and CldU are discriminated well by this procedure and can thus be used to study the dynamics of newly replicated DNA at the ultrastructural level (chapter 4).

To label entire chromosomes for EM studies, lower concentrations of thymidine analogues had to be used than are used for short pulses to label sites of replication. To obtain strong EM signals, we labeled chromosomes in vivo with BrdU. These studies showed that chromosomes are either separated by an interchromatin space or are apposed so closely that they appear to form one structure. Even so, chromatin intermingles at most over very small regions. Interchromatin spaces are also observed within chromosome territories. In these spaces, nuclear processes such as DNA replication and transcription are known to occur, thus bringing chromosomal structure and function together again. Details are given in chapter 5.

The experiments described in this thesis have changed our concept of chromosome territories from relatively compact units to well-organized domains, containing many channels in which nuclear processes take place. In chapter 6, we bring these findings together in a model that describes how chromatin may be organized within chromosome territories and discuss the implications for nuclear organization in general.
chapter 1

The study showed that the application of algorithms in machine learning, including the methods of decision trees, neural networks (Cormen, Leiserson, et al., 1997; 1995; Zeitel et al., 1993), can achieve excellent performance in complex problems with varying specific characteristics. As the core of artificial intelligence, neural networks are widely used in various fields such as text classification, speech recognition, and autonomous driving. We also explored the development of deep learning models which have achieved breakthroughs in some domains, such as image and speech recognition. In this dissertation, we introduce the mathematical models and algorithms that we developed in the modeling and analysis of our theoretical problems. The experimental results show the feasibility and efficiency of our models.