

## Ultrastructural cytochemical analyses of nuclear functional architecture

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For many years, ultrastructural cytochemistry has been a major tool for investigating the relationships between the structure and the functions of the cell nucleus. This article shortly reviews the contributions of transmission electron microscopy to the *in situ* studies of intranuclear distribution of chromatin domains and of essential nuclear functions, such as DNA replication, transcription and pre-mRNA processing. It attempts to analyse the role of different nuclear structural domains in nuclear functions, as well as further directions in this important field of research.

**Key words:** ultrastructural cytochemistry, nuclear functions, DNA replication, transcription, pre-mRNA processing.

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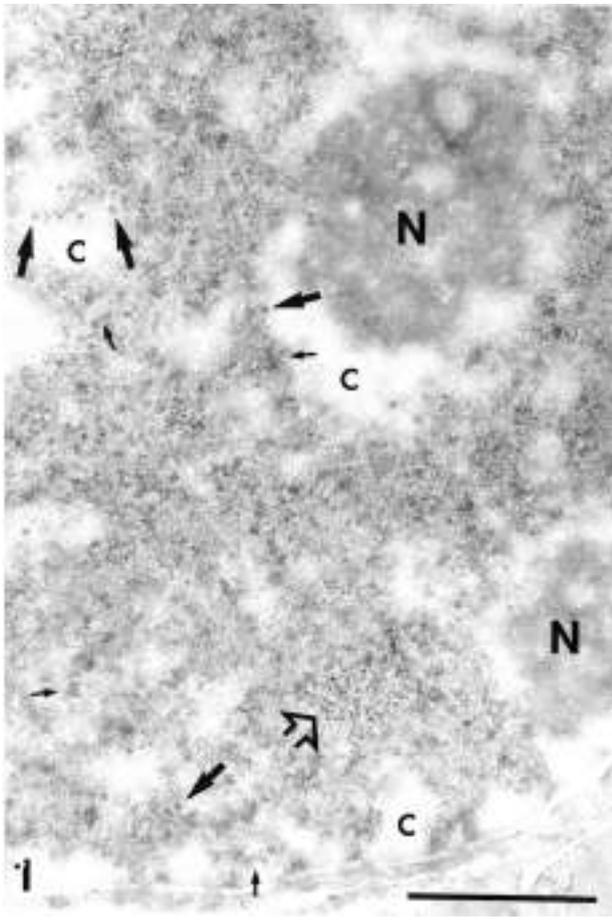
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Ultrastructural cytochemistry has been, during the last four decades, a powerful tool for investigating the morpho-functional relationships in the cell nucleus. Offering the possibility of high resolution analysis, its achievements created, long before the first confocal microscope became commercially available, a basis for further *in situ* investigations of nuclear functional architecture (see e.g. Swift, 1962; Bernhard and Granboulan, 1963; Smetana et al., 1963; Monneron and Bernhard, 1969).

Methods of transmission electron microscopic observation played an essential role in revealing structural features of molecular processes, such as RNA transcription (Miller and Beatty, 1969), assembly of DNA and histones into a chromatin fibre (Olins and Olins, 1974; Woodcock et al., 1976) and later also pre-mRNA splicing (Beyer and Osheim, 1988; Fakan et al., 1986; Osheim et al., 2002). At the cellular level *in situ*, ultrastructural cytochemistry has allowed one to describe and characterize a number of structural compartments or domains in the nucleus.

The internal architecture of the cell nucleus (Figure 1) consists of two major compartments, the nucleolus and the nucleoplasm. The nucleolus is the site of rRNA synthesis and processing, and ribosomal subunit assembly. It contains three main constituents: dense fibrillar component, fibrillar centers and granular component. The analysis of structure-function relationships in the nucleolus has been a subject of numerous, sometimes controversial reports (for more details about nucleolar functional morphology, see Goessens, 1984; Hozak, 1995; Biggiogera et al., 2001; Raska, 2003). In this short review article, we will concentrate on some morpho-functional aspects of major nucleoplasmic compartments or domains, especially those which have been investigated by means of methods of ultrastructural analysis of the nucleus *in situ*. General features concerning functional nuclear architecture



**Figure 1.** An ultrathin section of a rat hepatocyte nucleus after glutaraldehyde fixation and Epon embedding. Following the EDTA staining preferential for nuclear RNP constituents (Bernhard, 1969), nucleoplasmic structures such as perichromatin fibrils (some indicated by small arrows), perichromatin granules (large arrows) and clusters of interchromatin granules (open arrow) are easily visualized. Nucleoli (N) remain well contrasted, while chromatin (c) appears light grey. Bar represents 1  $\mu$ m.

have recently been reviewed elsewhere (Spector, 2003; Cremer and Cremer, 2001; Jaunin and Fakan, 2002) and will not be dealt with in the present article.

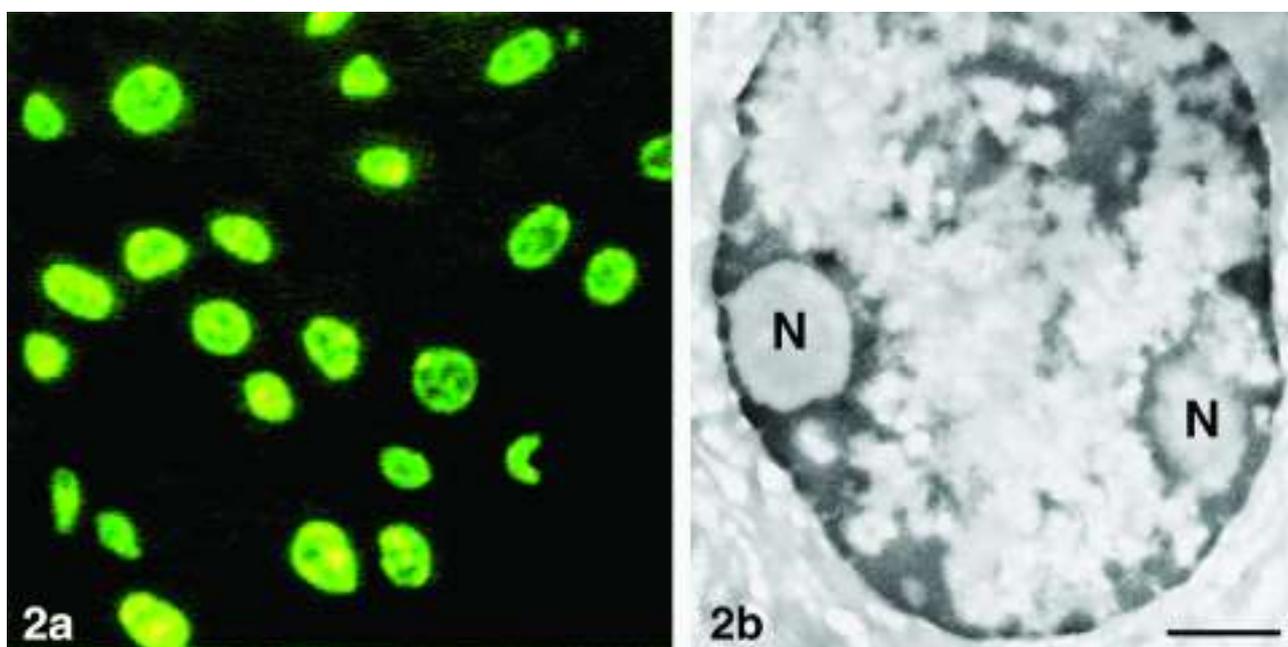
The nucleoplasm consists of chromatin areas represented mainly by condensed chromatin regions occurring either on the periphery of the nucleus adjacent to the nuclear lamina or throughout its interior. The nuclear area outside chromatin has been named the interchromatin space (e.g. Fakan and Puvion, 1980) or more recently also the interchromatin compartment (Cremer and Cremer, 2001) and it is largely devoid of DNA. There is a number of structural domains situated within the interchromatin space, most of them containing different kinds of RNA. Finally, an important nucleoplasmic domain, the perichromatin region, started

to emerge some thirty years ago, and has been confirmed as the major site of essential nuclear functions, such as DNA replication, transcription and probably also most steps of pre-mRNA processing.

### **Chromatin domains**

When chromatin is visualized at the light microscopic level by means of a DNA-specific fluorescence staining (Figure 2a) or of stably transfected histone-GFP constructs visualized in a confocal laser scanning microscope (CLSM) (see e.g. Cremer and Cremer, 2001), it appears as largely filling the nuclear volume. However, when DNA is specifically contrasted on ultrathin sections (Figure 2b), using a Feulgen-type procedure making use of osmium ammine (Cogliati and Gautier, 1973), the volume occupied by the chromatin looks much less extensive. This discrepancy is due to the difference in the thickness of the specimen examined, as well as in the resolution power (two orders of magnitude) between the two microscopic approaches. In effect, electron microscopic studies using reconstructions of serial section images showed that only 50 to 60% of the nuclear volume was occupied by condensed chromatin (Lopez-Velazquez et al., 1996; Esquivel et al., 1989), leaving the remaining volume to the interchromatin space and domains occurring throughout it.

The development of fluorescence *in situ* hybridization (FISH) techniques made it possible to localize territories of individual chromosomes in the interphase nucleus (for review, see van der Ploeg, 2000; Cremer and Cremer, 2001). This powerful approach has been largely applied and became a useful tool in biomedical research. However, when interpreting observations of fluorescence microscopy or CLSM, one must keep in mind the resolution limits of such microscopic methods (Stelzer, 1995). Moreover, FISH protocols mostly use denaturing conditions giving rise to a series of artifacts. When FISH treated specimens are further examined by electron microscopy (EM) extensive dispersion of DNA within the nucleus, leakage of DNA towards the cytoplasm and a general deterioration of the nuclear fine structure is observed (Solovei et al., 2002) This means that FISH methods, as mostly used so far, can hardly be applied to higher resolution analyses, such as, for instance, a precise localisation of a gene with regard to an interphase chromosome domain or territory. When applied at the EM level directly to ultrathin frozen



**Figure 2.** Mouse pancreas cell nuclei stained by the Feulgen type reaction giving rise to specific visualization of DNA. a. Acriflavine staining of a semithin section viewed in a fluorescence microscope. Micrograph by J. Fakan. b. Osmium ammine staining (Cogliati and Gautier, 1973) of an ultrathin frozen section. N= nucleoli, bar represents 1  $\mu$ m.

or resin sections under denaturing conditions using DNA probes, *in situ* hybridization (ISH) is often still too deleterious for the nuclear fine structure and the signal is not always sufficient.

To overcome these difficulties, Visser et al., (2000) have profited from the opportunity to label chromatin domains *in vivo* by means of BrdU, which can easily be detected, similarly to other halogenated precursors, by appropriate antibodies and immunoelectron microscopy (Jaunin et al., 1998). When cells, after an incubation with BrdU for a duration of one S-phase are further allowed to divide for several cell cycles, one can find, in the interphase nucleus, one to a few labeled chromatin domains obviously belonging to different chromosomes. This method has been very useful for analysing neighborhood topological relationships between labeled and unlabeled chromatin domains. Three major patterns have been observed: complete physical separation by interchromatin space, two domains appearing as one continuous chromatin area but exhibiting a sharp separation of label, and two domains appearing as one continuous area with a gradual transition of labeling density with chromatin fibres somewhat interspersed between the two domains, obviously thus allowing for chromosome-chromosome interactions. The interchromatin space formed interconnecting channels running

through and around chromosome domains or territories. Some of these features have been documented by reconstructions of small numbers of serial sections (Visser et al., 2000) However, compared to visualisation by FISH, the chromatin domains could not be attributed to specific chromosomes. An ISH method at the ultrastructural level, allowing for chromosome painting under conditions of a good ultrastructural preservation of the nuclear morphology still awaits further developments.

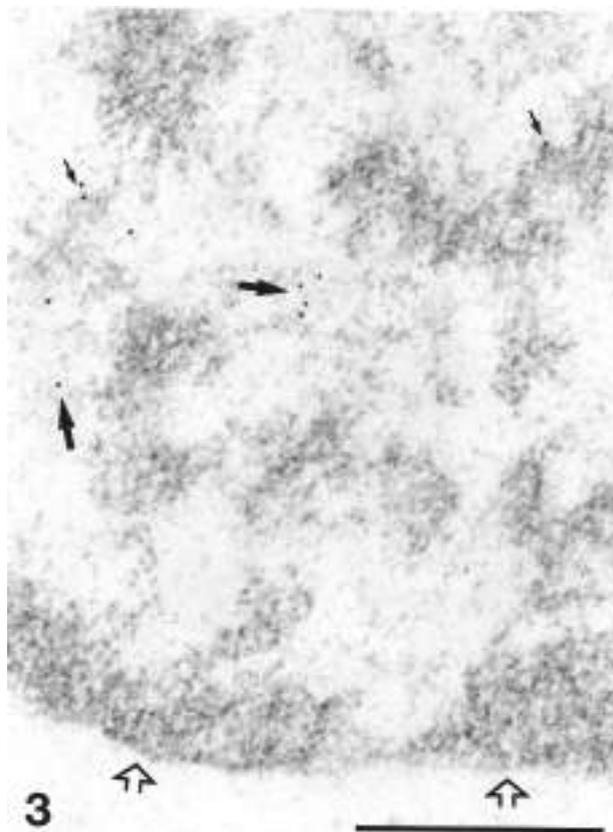
### **DNA replication**

Earlier high resolution *in situ* studies of DNA replication were carried out using  $^3\text{H}$ -thymidine as DNA precursor and EM autoradiography (reviewed e.g. in Fakan, 1978). Some of them clearly indicated that most DNA replication sites, visualized after very short labeling pulses of exponentially growing cells (Fakan and Hancock, 1974), occurred on the border of condensed chromatin areas. Later development of non-isotopic labeling procedures making use of halogenated deoxyuridines and immunocytochemistry with colloidal gold markers, allowed one to replace autoradiographic methods by a higher resolution approach. In addition to the resolution of ultrastructural methods necessary for a precise localization of replication sites, one must keep in mind the rapid rate of DNA replication (1-2

µm/min; Huberman and Riggs, 1968) thus requiring very short pulses with labeled precursors. As a result of such fine structural *in situ* investigations, several groups have shown that DNA synthesis predominantly occurs on chromatin fibres located on the periphery of condensed chromatin areas (Figure 3) (Sobczak-Thepot et al., 1993; Liu et al., 1995; Jaunin et al., 2000). This perichromatin domain contains dispersed chromatin regions and fibres, where nascent DNA was localized together with DNA polymerase  $\alpha$ , cyclin A and PCNA (Sobczak-Thepot et al., 1993; Jaunin et al., 2000). Moreover, when the kinetics of newly synthesized DNA was analyzed on ultrathin sections of chinese hamster V79 cells stained with osmium ammine (Cogliati and Gautier, 1973) to specifically visualize DNA, fractions of DNA synthesized during a short period of time were subsequently rapidly internalized into the condensed chromatin areas from their periphery. Such movement of DNA during the replication process was further confirmed by pulse-chase experiments making use of differential visualization of two halogenated precursors (I- and Cl-labeled) incorporated during different successive periods of time separated by unlabeled chase period (Jaunin et al., 2000). All these experiments therefore suggest that DNA is, during the synthetic period, rapidly moving between the replication sites occurring within the peripheral perichromatin region of chromatin domains and their interior. This replication pattern, although studied so far mainly on exponentially growing unsynchronized cells, seems to be independent of the S-phase period. In early S-phase, chromatin occurs rather dispersed in numerous spots, whereas cells in late S-phase show labeling on larger more condensed domains (O'Keefe et al., 1992; Leblond and El-Alfi, 1996; Jaunin et al., 1998). Observations obtained by electron microscopic studies have recently been compared with models of *in situ* DNA replication provided by immunofluorescence and confocal imaging and the different approaches have been critically evaluated (Jaunin and Fakan, 2002).

### **Transcription sites**

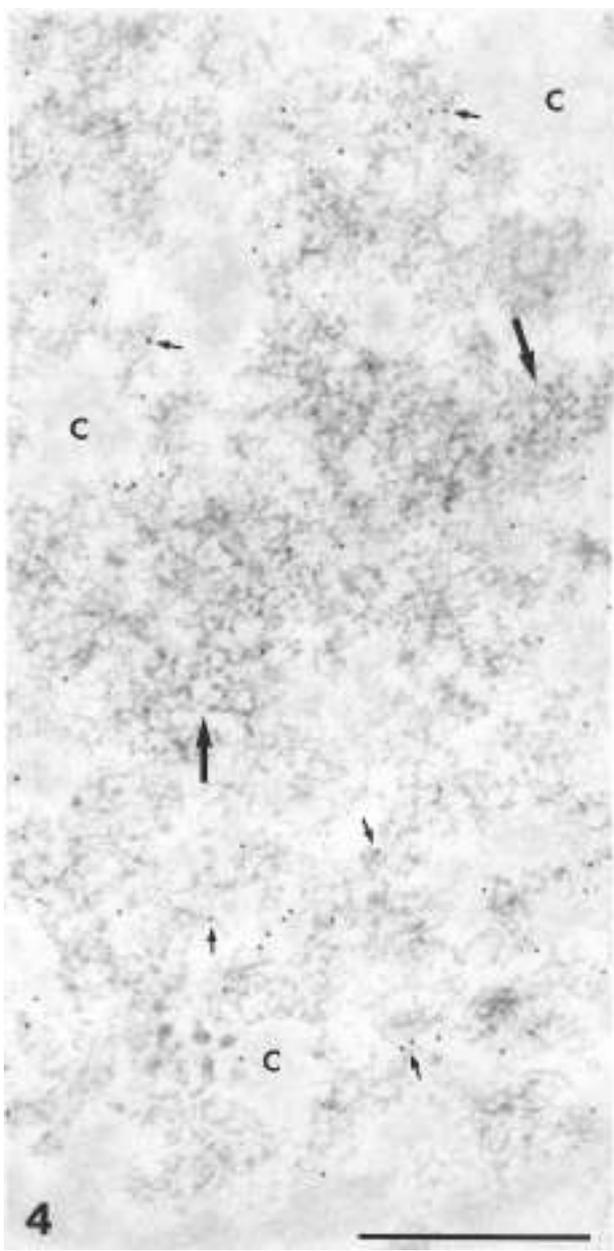
Methods of ultrastructural cytochemistry have played a major role in investigating and visualizing the *in situ* organisation of transcription with regards to different structural domains of the cell nucleus. Earlier work made use of tritium labeled RNA precursors and high resolution autoradiogra-



**Figure 3.** Chinese hamster V79 cell labeled for 2 min with BrdU, fixed in paraformaldehyde and embedded into Lowicryl K4M resin. Ultrathin section stained with osmium ammine specific for DNA. Label occurs on the periphery of condensed chromatin (small arrows) sometimes associated with dispersed chromatin fibres (large arrows). Open arrows indicate nuclear periphery. For more details see Jaunin et al. (2000). Micrograph by F. Jaunin. Bar represents 0.5 µm.

phy (for review see Fakan, 1978). This approach, in combination with a contrasting method preferential for nuclear ribonucleoprotein structural constituents (Bernhard, 1969), allowed one to investigate with more precision the intranuclear distribution of pre-mRNA transcription sites and pointed out the perichromatin regions as major sites of transcription (Fakan and Bernhard, 1971). The application of this contrasting method was essential for describing a new RNP containing nuclear component, the perichromatin fibrils (Figure 1), which previously could not be clearly differentiated from chromatin (Monneron and Bernhard, 1969). Further, EM autoradiographic analyses revealed perichromatin fibrils as structures containing rapidly labeled RNA synthesized within the perichromatin region (Nash et al., 1975; Fakan et al., 1976), representing *in situ* forms of hnRNA transcripts (Fakan, 1994).

The development of antibodies specific for differ-



**Figure 4.** Rat HTC cell fixed in paraformaldehyde 10 min after microinjection of BrUTP, and embedded into LR White resin. Ultrathin section stained with the EDTA procedure. Gold particles mostly occur on the periphery of condensed chromatin regions (c), mainly associated with perichromatin fibrils (some indicated by small arrows). Clusters of interchromatin granules (large arrows) are virtually devoid of label. For more details see Cmarko et al. (1999). Micrograph by D. Cmarko. Bar represents 0.5  $\mu\text{m}$ .

ent nuclear components and, later on, the introduction of non-radioactive labeling methods of RNA by means of brominated precursors, opened a new field of high resolution investigation of nuclear structural domains by immunoelectron microscopy. The application of monoclonal antibodies directed against hnRNP core proteins (Leser et al., 1984)

demonstrated these antibodies mostly associated with perichromatin fibrils and granules, while the interchromatin granule clusters and Cajal (coiled) bodies were virtually devoid of hnRNP proteins (Fakan et al., 1984). CLSM analyses of cells shortly labeled with Br-UTP (Wansink et al., 1993; Jackson et al., 1993) revealed the fluorescence signal either in the form of multiple tiny spots or diffuse labeling scattered throughout the nucleoplasm. When directly visualized on ultrathin sections of cells microinjected with Br-UTP (Cmarko et al., 1999) or after Br-uridine incubation of cells (Trentani et al., 2003), the EM immunogold signal clearly revealed the perichromatin fibrils as main incorporation sites of the precursor and the perichromatin region as the major transcription domain in the nucleoplasm (Figure 4), thus confirming previous autoradiographic findings.

Perichromatin fibrils occurring within the perichromatin region are also main sites of the localization of transcription factors such as RNA polymerase II or TFIIF (Cmarko et al., 1999). Br-labeled RNA in perichromatin fibrils following short incubation periods is mostly represented by individual gold particles. Moreover, specific staining for RNA by means of terbium citrate (Biggiogera and Fakan, 1998) confirms that gold particles indeed associate with the RNA of the fibrils (Trentani et al., 2003). These observations reveal that pre-mRNA transcription mostly occurs in multiple individual transcription sites rather than within *transcription factories* (Iborra et al., 1996; Jackson et al., 1998) or complex *transcription sites* shown as structurally ill-defined nuclear regions (Jackson, 2003).

### **Pre-mRNA processing**

Perichromatin fibrils have been reported as accumulating all pre-mRNA processing or processing-associated factors studied so far. These include snRNPs (Fakan et al., 1984; Puvion et al., 1984), m3G cap structure of snRNAs (Malatesta et al., 1994a; Trentani et al., 2003), SC-35 protein (Spector et al., 1991), poly(A) polymerase (Cmarko et al., 1999) and survival of motor neuron protein (SMN) (Malatesta et al., 2004). Moreover, ISH assays with a biotinylated oligo-dT probe revealed the occurrence of poly(A)-containing RNA in perichromatin fibrils (Visa et al., 1993a). Consequently, since perichromatin fibrils are the only nucleoplasmic structural component containing

all transcription and processing factors studied until now, it is obvious that they also represent the site where most pre-mRNA processing steps take place.

Perichromatin granules, another structural constituent observed in the perichromatin regions, are almost exclusively limited to this nucleoplasmic domain. They mostly occur individually and can occasionally be observed as winding from a perichromatin fibril. Their structure and cytochemical properties, as well as the arrangement of RNA within perichromatin granules, look similar to Balbiani ring granules in *Chironomus polytene* nuclei (Vazquez-Nin et al., 1996, 1997a). The latter granules have been shown to represent an intranuclear transport form of large pre-mRNA molecules. Splicing factors have been shown to associate co-transcriptionally with Balbiani granules in the process of their formation (Vazquez-Nin et al., 1990), while free granules contain processed RNA (for review, see Daneholt, 2001). As the number of perichromatin granules correlated with the rate of RNA export in target cells of hypophyseal hormones (Vazquez-Nin et al., 1997b), the granules obviously represent intranuclear storage and transport means of pre-mRNA in mammalian cell nuclei.

In the interchromatin space, two remarkable domains can be observed in mammalian and many other higher eukaryotic cells: interchromatin granule (IG) clusters and the Cajal (coiled) bodies (CBs). Previously characterized especially by means of basic cytochemical methods (Monneron and Bernhard, 1969), they both contain RNA. Original ultrastructural immunocytochemical methods showed that IG accumulated snRNPs (Spector et al., 1983; Fakan et al., 1984; Puvion et al., 1984), the splicing factor SC-35 protein (Spector et al., 1991) and the m<sup>3</sup>G cap of snRNAs (Malatesta et al., 1994a; Trentani et al., 2003). Moreover, they have been virtually devoid of newly synthesized RNA (Fakan and Bernhard, 1971; Cmarko et al., 1999; Trentani et al., 2003) and of hnRNP core proteins (Fakan et al., 1984). However, ISH experiments with oligo-dT probes allowed Visa et al. (1993a) to reveal some poly(A) RNA in IG in addition to perichromatin fibrils. The clusters of granules can be specifically detected by means of antibodies against marker proteins such as the PANA protein (Clevenger and Epstein, 1984) and were later also named *speckles* by light microscopists. A detailed fluorescence microscopic quantitative digital analysis of nascent Br-RNA,

SC-35 protein and poly(A) distribution in nuclei of cultured cells revealed that, for all probes examined, most of the signal was diffusely spread throughout the nucleoplasm (Fay et al., 1997). This was also striking for SC-35 labeling, which was especially diffusely distributed throughout the nucleoplasm (70-80%), while only a minority was concentrated in IG clusters (speckles). This clearly argues that SC-35 is not a marker factor of speckles, as erroneously supposed in a number of fluorescence microscopic studies. Taking advantage of *in vivo* visualization of overexpressed SF-2 splicing factor in cells transfected with a GFP construct, Misteli et al., (1997) made an elegant demonstration by time laps microscopy of the splicing factor delivery from speckles towards transcription sites. All these data speak in favor of IG clusters (speckles) as sites of accumulation of splicing factors and possible pre-assembly of spliceosome components. Although some poly(A) RNA was identified in this compartment, the absence of poly(A) polymerase, rapidly labeled RNA, as well as of hnRNP core proteins rules out IGs as sites of transcription or major pre-mRNA processing. It also questions the type of poly(A) containing RNA fraction occurring in IG clusters and the reason for its presence in this nuclear domain. A recent study aiming at examining the involvement of IG clusters in transcription and pre-mRNA splicing used the serine-arginine (SR) protein kinase cdc2-like kinase Clk/STY to manipulate the integrity of IG clusters *in vivo*. Both immunofluorescence and transmission electron microscopy showed that overexpression of Clk/STY leads to dispersion of interchromatin granules in the nucleoplasm, while an inactive mutant did not do so. These observations, suggesting that hyperphosphorylation of SR proteins gives rise to IG dispersion, also showed that cells lacking IG clusters continued to synthesize nascent pre-mRNA. However, accumulation of splicing factors at sites of pre-mRNA synthesis, as well as splicing were strongly reduced, showing that IG clusters integrity was important for coordination of transcription and pre-mRNA splicing (Sacco-Bubulya and Spector, 2002). A domain, named IG associated zones and containing U1, but not U2 snRNA, can be observed in close proximity of IG clusters (Visa et al., 1993b). It was shown to contain moderate amounts of newly synthesized RNA after longer labeling periods, as well as some transcription factors (Cmarko et al., 1999) and SMN protein (Mala-

testa et al., 2004). The functional role of this domain remains unclear.

The CB is another nucleoplasmic domain accumulating snRNPs but not SR rich splicing factors (see Gall, 2000, for review). First examined by ultrastructural cytochemistry by Monneron and Bernhard (1969), it appears to be a dynamic structure. EM studies showed its abundance in nuclei of different cell types during dormouse hibernation in both the nucleolus and the nucleoplasm (Malatesta et al., 1994 a, b). Surprisingly, most CBs dissociated rapidly during the arousal from hibernation, their marker protein p80-coilin being detectable until the complete disappearance of their structural components. *In vivo* fluorescence microscopic studies making use of a cell line stably expressing a p80-coilin-GFP fusion protein suggested a relatively high level of movement of CBs within the nucleoplasm, as well as between nucleoplasm and nucleoli (Platani et al., 2000). Recent work also examined the structural relationships between CBs and a domain named electron-dense fibro-granular clusters (EFGCs), which accumulated SMN protein and Gemin2 and obviously corresponds to the domain originally described by immunofluorescence microscopy as gems (Liu and Dreyfuss, 1996). CBs and EFGCs were sometimes observed in close association, but usually distant from each other (Malatesta et al., 2004). Both domains seem to be related to splicing factor storage and assembly, but their precise roles in nuclear functions still remain to be elucidated.

Another nucleoplasmic domain, the promyelocytic leukemia (PML) nuclear body can be relatively frequent in some cells. Ultrastructurally appearing as a round domain consisting of a peripheral layer and a finely fibrillar core (Koken et al., 1994), it contains numerous proteins and many potential functions were proposed for its role in the nucleus (for review see Borden, 2002). Interestingly, one class of PML bodies was shown to move in a metabolic-energy-dependent manner, possibly involving nuclear myosin (Muratani et al., 2002).

### **Conclusions and perspectives**

Most of the points discussed above confirm the importance of ultrastructural cytochemistry in investigating the functional nuclear architecture. Although providing a two-dimensional image, transmission electron microscopy in combination with various cytochemical methods continues to be an

invaluable tool in analyses of structure-function relationships in the cell especially thanks to resolving power superior to light microscopy. In addition, many LM cytochemical methods require harsh treatments of the cell, such as permeabilisation and denaturation, which can lead to a loss or displacement of protein or nucleic acid nuclear constituents. Live cell microscopy, which has recently been an object of important rapid developments (see e.g. Janicki and Spector, 2003; Zink et al., 2003), when used in combination with subsequent ultrastructural analysis of the same cell, certainly represents one of the major directions in this field of research. Most studies on DNA replication and transcription substantiate the importance of the perichromatin region as a functionally essential domain in the nucleus. The analyses of transcription sites indicate that the majority of active genes are located in this domain. Information about the diffusion of relatively large molecules or molecular complexes into compact nuclear compartments, such as condensed chromatin areas (Verschure et al., 2003) indicates that the organisation of transcriptionally active regions may not be dependent on accessibility problems. Recent observations that polycomb group gene silencing proteins are concentrated in this nuclear domain (Cmarko et al., 2003) further suggest that some epigenetically silenced genes are also localized in the perichromatin regions. Domains located in the interchromatin space, such as IG clusters and CBs obviously play an important role in accumulation/storage of pre-mRNA processing factors and their delivery towards sites of pre-mRNA synthesis and processing. CBs appear to be dynamic structures, but we do not know yet how they are generated and what the relationship is or whether there is a difference between those observed in the nucleolus and the ones occurring in the nucleoplasm. The nuclear matrix, an operational term for residual intranuclear fibrogranular essentially protein network, was proposed as a scaffolding structure. However, there is no clear evidence, so far, about the existence of such a structural network in the intact nucleus *in situ* (for more details, see Hancock, 2000; Pederson, 2000). As to the internal organization of the nucleus, major information is still lacking. It is possible that nuclear architecture is self-organizing (Misteli, 2001) and that some small nuclear domains, although structurally relatively complex, are generated *de novo* as they also dissociate in the

nucleoplasm (Malatesta et al., 1994a). A special case is the formation of heterogeneous ectopic RNP-containing structures occurring in nuclei which undergo experimental or natural transcription arrest (Biggiogera and Pellicciari, 2000). A series of reports about the intranuclear occurrence of lamins (Hozak et al., 1995), actin (review in Shumaker et al. 2003) and myosin (Pestic-Dragovich et al., 2000) brought into question the role of these proteins in nuclear functions and in a more general sense in the nuclear functional architecture. Moreover, the abundant non-protein-coding RNA observed in higher eukaryotes may not only play a role in controlling mRNA turnover and developmental timing of protein expression but can also be involved in transcription and splicing processes and, in a broader sense, in the functional architecture of the nucleus (for review see Mattick, 2003). Furthermore, an essential issue to be investigated in the future, will be the way how molecules move in the nucleus.

The export mechanisms of RNA and, in particular, of mRNA, with regards to nuclear compartments have been studied for some time and a free diffusion model seems to be a prevalent one for RNA movement throughout the nucleoplasm (for more details, see Kramer et al., 1994; Singh et al., 1999; Politz and Pederson, 2000). Another important issue is the recruitment of factors involved in RNA synthesis at the beginning of interphase of daughter cells after mitosis and following nuclear envelope/lamina reformation. According to work from Spector's group, pre-mRNA transcription and processing factors are detected in the nucleus in a non-random and sequential manner favoring the idea that the entry of the RNA polymerase II gene expression machinery is staged and ordered (Prasanth et al., 2003).

In conclusion, a number of important questions regarding the morpho-functional aspects in the cell nucleus are to be studied and the complementarity of different high standard microscopic approaches will be fundamental for obtaining sufficiently complete and significant answers.

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