Hypothesis for the influence of fixatives on the chromatin patterns of interphase nuclei, based on shrinkage and retraction of nuclear and perinuclear structures

L. P. BIGNOLD
Division of Tissue Pathology, Institute of Medical and Veterinary Science, PO Box 14, Rundle Mall, Adelaide, SA 5001, Australia.

Accepted: 21 January 2002

ABSTRACT

Nuclear chromatin patterns are used to distinguish normal and abnormal cells in histopathology and cytopathology. However, many chromatin pattern features are affected by aspects of tissue processing, especially fixation. Major effects of aldehyde and/or ethanol fixation on nuclei in the living state include shrinkage, chromatin aggregation and production of a 'chromatinic rim'. The mechanisms of these effects are poorly understood. In the past, possible mechanisms of fixation-induced morphological change have been considered only in terms of the theoretical model of the nucleus, which involves only a random tangle of partly unfolded chromosomes contained within the nuclear membrane. Such a model provides no basis for chromatin to be associated with the nuclear envelope, and hence no obvious clue to a mechanism for the formation of the 'chromatinic rim' in fixed nuclei. In recent years, two new models of nuclear structure have been described. The nuclear membrane-bound, chromosomal-domain model is based on the discoveries of chromatin-nuclear membrane attachments and of the localisation of the chromatin of each chromosome within discrete, exclusive parts of the nucleus (the 'domain' of each partly unfolded chromosome). The nuclear matrix/scaffold model is based on the discovery of relatively insoluble proteins in nuclei, which it suggests forms a 'matrix' and modulates gene expression by affecting transcription of DNA. Here, a hypothesis for fixation-associated chromatin pattern formation based mainly on the first model but partially relying on the second, is presented. The hypothesis offers explanations of the variations of appearance of nuclei according to fixation (especially air-drying versus wet-fixation with formaldehyde, glutaraldehyde or ethanol); the appearances of the nuclei of more metabolically active versus less metabolically active cells of the same type; the appearances of nuclei after fixation with osmium tetroxide; and of the marked central clearing ('egg-shell' or 'orphan Annie' appearance) of tumour nuclei of papillary carcinoma of the thyroid gland. A similar process may underlie the phenomenon of 'chromatin margination' seen in apoptosis. Various tests of the hypothesis, such as time-lapse confocal microscopy of living nuclei during fixation, are suggested. The significance of the theory is that it suggests that chromatin patterns could be investigated in terms of qualitative and quantitative aspects of nuclear components, and hence be related to the results of studies of the structure and function of nuclei in health and disease.

KEY WORDS: Chromatin. Hardness. Nuclear membrane. Tissue fixation.

Introduction

Nuclei were described by Leewenhoek in 1700 but named by the botanist Robert Brown in 1831.[1] By the middle of the nineteenth century, nuclei were thought to be composed of different substances to those of cytoplasm, and to be an essential feature of cells that exhibited ongoing viability.[2] The discovery, in the late nineteenth and early twentieth centuries,[3] of genes and their transmission from one cell generation to the next by mitosis and meiosis added storage and propagation of heredity to known functions of the nucleus.

Effects of fixation on nuclear appearance

From the beginnings of microscopy of fixed, sectioned and stained tissue, and especially after the introduction (in 1893) of formaldehyde fixation,[4] it has been recognised that nuclear chromatin usually appears more coarsely clumped and more peripherally distributed in cells processed histologically compared to appearances in the living state.[4-7] These discrepancies have been confirmed by phase contrast microscopic studies (begun in the 1940s), which demonstrate a homogenous distribution of chromatin, with little aggregation, in living cells.[5,6] Maximow and Bloom[5] provided illustrations of nuclei prepared by many different histological techniques. Baker[7] described extensive studies of living tissue-cultured cells that were continuously observed microscopically during fixation, noting in particular that ethanol caused a 'coarse coagulum' to appear in nuclei.

Cells of differing types have nuclear chromatin patterns that vary more markedly after aldehyde/ethanol fixation. Thus, the nuclei of mucus-producing intestinal cells have large, pale-staining central zones, plasma cells have coarse

Email: leon.bignold@adelaide.edu.au

radial aggregates of chromatin ('clock-face' or 'cart-wheel' pattern), and hepatocytes show intermediate patterns. Additionally, chromatin patterns after fixation vary between metabolically more active and less active examples of the same cell type. Nuclei of less active cells are small and darkly-staining, as in lymphocytes, fibrocytes, and osteocytes, while the nuclei of their more active (activated or blast) forms are larger and have less densely staining central zones.

The appearances of chromatin patterns in cytological smears are similarly variable according to the manner of processing.[8,9] For example, smeared cells which are air-dried alone before staining, have large nuclei with little chromatin aggregation. Post-fixation with methanol (as in the Diff-Quik or May Grunwald-Giemsa methods) provides some 'sharpening' of chromatin pattern. However, the greatest clarity of chromatin pattern is achieved by fixation with pure ethanol in the wet state (as in the Papanicolaou method). This method is associated particularly with marked overall shrinkage of the cell and nucleus.[6,8,9] Bibbo,[6] provides good illustrations of the differing appearances of leukaemic cells prepared by Diff-Quik versus Papanicolaou methods.

Among malignancies, cell-to-cell chromatin pattern variation is a feature of tumour nuclei, although the mechanisms of these abnormalities rarely have been studied. In papillary carcinoma of thyroid gland, a characteristic pronounced central clearing ('egg shell' or 'orphan Annie' appearance) of the nuclei occurs after histological processing. However, these changes are not demonstrable in frozen sections and air-dried imprint preparations. Rosai *et al*.[10] state that this clearing of the central nuclear zone is induced by fixation and implies 'some intrinsic alteration of the chromatin structure or associated nuclear proteins'.

Finally, differences in the appearances of chromatin also occur in electron microscopy. Fixation with osmium tetroxide results in nuclei (including those of plasma cells) showing little chromatin aggregation.[11,12] Formaldehyde produces some chromatin condensation in electron micrographs, but the sharpest chromatin aggregations are achieved by fixation in glutaraldehyde.[11]

General effects of fixatives on tissue structures and chromatin

The general effects of fixatives are relevant to the consideration of how fixation might affect nuclear appearance. In general, a major function is inactivation of autolytic mechanisms, and thus they must alter at least the structure of the substrate-binding sites of the enzymes responsible. A second desirable feature of fixation is to render proteins, along with as many other tissue materials as possible, insoluble in water and the other solvents used in histological processing.[7-9] Both events require denaturation of the native structure. Known mechanisms of this denaturation include i) formation of chemical bridges between adjacent molecules (aldehydes), mainly between amino groups of proteins (of either the same or adjacent polypeptide chains) but protein-DNA bridging can occur under some conditions;[13] ii) coagulation, especially by dehydration (ethanol); and iii) interaction, or oxidation, and even precipitation with heavy metals (chromium, osmium, mercury).

While these general chemical roles are known, most fixatives have many different actions, which can depend not only on different physiochemical aspects of 'target' structures but also on ambient conditions such as temperature, pH, ionic conditions and duration of exposure. Therefore, precise effects of fixatives, when applied to the complex and variable mixtures of diverse macromolecules that comprise nuclei, have been difficult to establish.

Notwithstanding the above, many fixatives – and particularly ethanol – shrink and harden biological structures.[7-9] Shrinkage and hardening of any particular structure is likely to depend not only on the particular fixative being used but also on qualitative aspects of the component proteins.

For example, the proportion of amino acids with groups suitable for bridging and the degree of native hydration before fixation and dehydration are probably relevant. In addition, the overall concentration of the protein component is important for the final degree of hardness, because hardness is likely to vary directly with the protein concentration in the available volume. Baker[7] found that, with the exception of acetone, ethanol produces the greatest degree of hardening.

It is likely that this diversity of effects applies to chromatin to a great degree, since the proteins associated with DNA presumably are similarly susceptible to the effects of fixatives as are other proteins, and direct protein-DNA bridging may occur. The juxtaposition of a negatively-charged macromolecule, held in a double helix by hydrogen bonds, may well increase the range of possible chemical 'targets' for the fixatives. All fixatives can be considered as adverse factors that are likely to induce the collapse of previously-dispersed chromatin in varying degrees according to chemical nature.

Composition of nuclei and structure of chromatin relevant to fixation and histological appearance

Early quantitative biochemical studies from the 1940s to 1960s showed that, excluding the nucleolus, nuclei consist almost entirely of virtually fixed quantities (for each species) of DNA and histone proteins, together with variable quantities of water, RNA and diverse non-histone proteins.[8]

As a group, non-histone nuclear proteins increase in overall quantity per nucleus (along with water content) with cell activation.[8] Recently, these proteins have been demonstrated to show cell type-specific patterns when examined by two-dimensional electrophoresis.[14-16] Therefore, electrophoretic patterns of non-histone nuclear proteins, together with RNA content, represent the major compositional difference between types of nuclei that have differing histologically appearances.

The structure of chromatin (the stainable material in nuclei) has been studied extensively, and is known to consist of a basic 30 nm 'sinusoidal fibre'[17,18] that, during interphase, is compacted to 10-20% of the degree of compaction of chromosomes in metaphase.[19] However, detailed knowledge of this chromatin compaction during interphase (as opposed to the more pronounced folding which occurs during formation of chromosomes) is lacking.[20-22]

Another aspect of chromatin structure during interphase is that it is liable to non-specific collapse (aggregation) under adverse conditions, during which it becomes sticky.[23] Sometimes, this injury-induced collapse can be reversible[24] but usually it is associated with irreversible inactivation and loss of cell viability. More recently, aggregation of chromatin following cell injury has been described as part of apoptosis.[25] Of relevance to the current hypothesis is that movement of compacted chromatin to the periphery of the cell ('chromatin margination'[26,27]) during apoptosis is well documented but not explained.

Models of nuclear structure *in vivo*

Throughout the whole period of investigation of the composition of nuclei, there has been controversy about whether or not the discovered components provide nuclei with any internal structure to assist their metabolism-altering and heredity-propagating functions, and, if so, what it might be.

'No internal structure' model

Initially, studies of the biophysical properties of nuclei *in vivo* were conducted by manipulation and injection experiments using thin mechanical probes and needles. Nuclei were found to be freely moveable in cytoplasm, and neither this movement nor temporary distortions of nuclear shape had any apparent ill-effect on the viability of the individual cell.[28]

Penetration of nuclei by fine probes permitted manipulation of the nucleolus within the nucleus, without causing any distortion of the overall nuclear outline. This suggested that no large, solid or rigid intranuclear structures exist *in vivo* and, especially, no such structures exist that are attached to the nuclear membrane. However, penetration of nuclei did cause subsequent collapse (granular precipitation) of chromatin, with chromatin sticking to the microneedle. This was followed by shrivelling of the whole nucleus, and death of the cell.[28]

The most commonly cited model of nuclear structure, therefore, has been of a flexible and slightly elastic bag (nuclear membrane or 'envelope') containing a random tangle of partially unravelled chromosomes,[29] together with histone proteins, non-histone proteins, water and some RNA.

Nuclear membrane-bound chromosomal-domain model

This model relies on two assertions: (1) that most chromosomes are focally attached to the nuclear membrane, and (2) that chromosomes in interphase occupy their own individual, more or less exclusive space in the nucleus.

With regard to membrane-binding of chromatin *in vivo*, early supportive evidence included the fact that chromatin can be observed condensed inside the nuclear membrane in some living cells.[23] The model was given additional support by the discovery (in the 1940s) that the Barr body is a folded X chromosome, and is usually bound to the inner aspect of the nuclear membrane, in a constant relative position depending on cell type.[23]

During the same period, investigations of cell organelles fractionated by ultracentrifugation began.[30] Often, these studies involved the use of various salt solutions to dissociate the cell components and then density gradient ultracentrifugation (using sucrose) to separate them as 'fractions'. Electron microscopy was then carried out to identify the structures in each fraction. Methods for separating nuclear membranes usually resulted in fractions that contained nuclear membranes mixed with part of the nuclear DNA. To remove this DNA, high salt concentrations and DNase were used,[31] implying strong binding between the DNA and nuclear membrane *in vivo*. Moreover, if nuclei are ruptured during the preparative processes, chromatin outside the nucleus sticks to various cytoplasmic organelles and structures, including plasma membrane.[31] More recently, the discovery of lamins has supported the existence of strong focal DNA binding to the region of the nuclear membrane.

Whether or not the centromeres (those parts of the chromosomes to which the spindle attaches in metaphase) are associated with the nuclear membrane is controversial. Studies using immunoperoxidase techniques with anti-centromere antibodies suggest that the centromeres of most chromosomes (especially the larger ones) are attached to the inner nuclear membrane, while the remainder have their centromeres located randomly through the centre of the nucleus.[32] However, other authors have provided evidence that the centromeres are not particularly associated with the nuclear membrane but are uniformly distributed throughout the nucleus.[33]

Evidence for the existence of specific, mutually exclusive chromosomal domains in the nucleus (rather than the chromatin of the chromosomes being mixed together) has been obtained over the last 20 years by fluorescence *in situ* hybridisation (FISH).[34] This method involves the use of complementary DNA sequences labelled with a fluorescent dye to locate specific DNA sequences within nuclei. It can be used on cell smears and even living cells. Such studies have shown that the unfolded chromosomes occupy discrete (non-overlapping) domains that collectively fill the whole interior of the nucleus during interphase.[17,35-37]

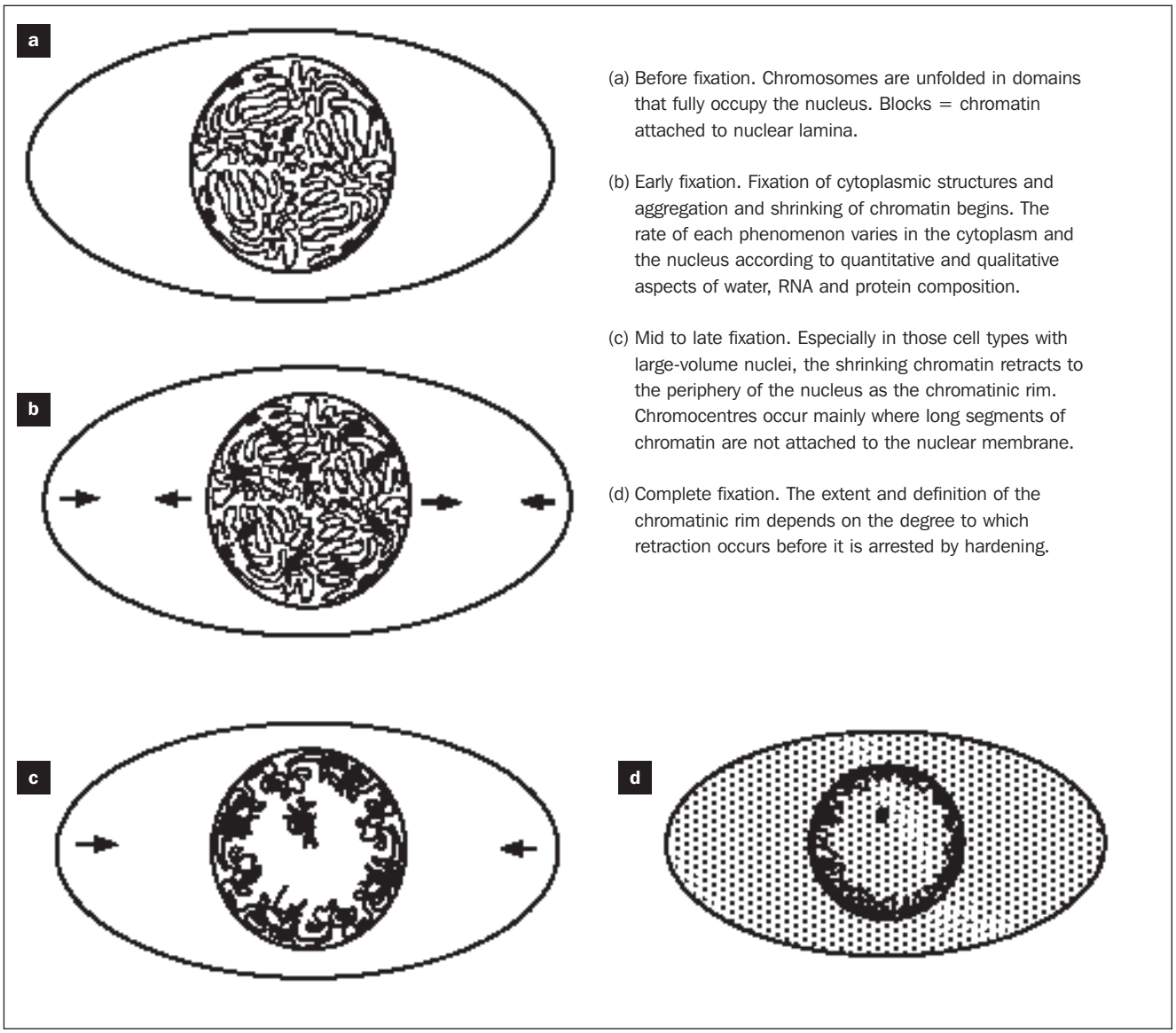
Nuclear matrix/scaffold model

Differences between one cell type and another are largely the result of differing patterns of gene expression. Attempts to identify the mechanisms of gene expression regulation have included the biochemical isolation and characterisation of the non-histone proteins of the nucleus in the search for 'transcription factors' and other gene expression-controlling proteins. However, these studies have also identified relatively insoluble types of nuclear proteins that are sometimes referred to as nuclear 'matrix' proteins.[38-40] Together with nuclear membrane proteins (especially lamins) and the nucleolar matrix proteins, these are thought to form the nuclear 'scaffold'.[32]

The nuclear matrix or scaffold has been suggested to function as an internal skeleton, to which transcription-regulating proteins and/or DNA itself might become attached, so that gene transcription can be controlled.[38-40] Nevertheless, many of the findings on which these suggestions are based have been attributed to preparative artefacts of various types.[41]

Lamins are one particular family of intranuclear fibrous proteins[42] that are located particularly in the submembranous region of the nucleus (also referred to as the 'nuclear lamina'). Lamins are thought to have a structural role, giving the periphery of the nucleus mechanical strength[43] and

Fig. 1. Model of nuclear appearances induced by fixatives, especially aldehydes and ethanol. A fine diffuse chromatin pattern occurs when the nucleus hardens early in chromatin shrinkage/retraction. A coarse chromatin pattern occurs when the nucleus hardens after chromatin shrinkage and retraction are well advanced.



continuity with the cytoskeleton.[40] Lamins are known to bind chromatin and certain DNA sequences, and thus may have a role in the control of gene expression.[44]

Hypothesis for the changes of nuclear appearances according to fixation and processing

The hypothesis advanced here is that chromatin patterns produced following histological processing with aldehydes and ethanol substantially are the result of fixative-induced chromatin aggregation and shrinkage. The extent to which these processes operate in individual situations is modified by the diversity of surrounding substances (mainly water, non-histone proteins and RNA), as well as the type of fixative and conditions of fixation employed. Retraction of chromatin to the periphery of the nucleus follows aggregation and shrinkage because of the attachment of the chromatin of unfolded chromosomes to the nuclear lamina.

Fundamental proposal (Fig. 1)

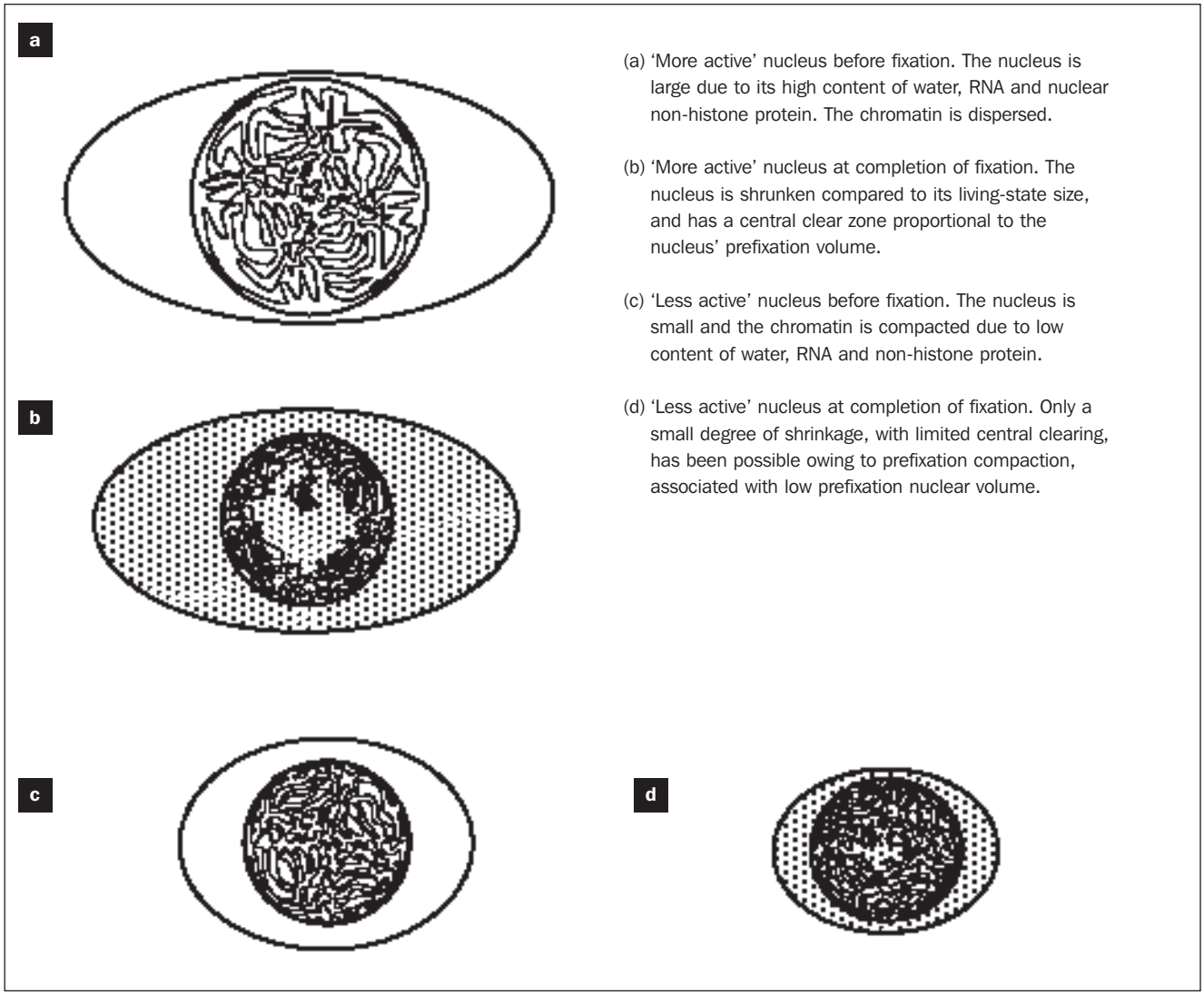
The hypothesis for the appearance of nuclei in aldehyde- and ethanol-fixed and processed preparations comprises the following suppositions:

1. In the prefixed state, the periphery of the nucleus is more rigid than the centre, probably due to the subnuclear membrane location of lamins. Focally, chromatin is bound to the inner nuclear lamina.

2. As the fixatives penetrate the perinuclear cytoplasm and nucleus, aggregation of aggregation-susceptible substances and shrinkage of shrinkage-susceptible substances occur.

3. Aggregation of chromatin occurs rapidly because of the

Fig. 2. Comparison of 'more active' and 'less active' nuclei.



sensitivity of its dispersed state during interphase to collapse under adverse influences.

4. Once formed, chromatin aggregates are liable to shrink, depending on ambient conditions (especially water, RNA and nuclear non-histone protein content).

5. As foci of chromatin from most of the chromosomes are attached to the rigid periphery of the nucleus, and the unattached parts are free in the softer central zone of the nucleus, shrinkage causes the chromatin aggregates to move towards (i.e. retract to) the periphery of the nucleus. Attachment of chromatin to the nuclear lamina may be enhanced as an effect of fixative-induced 'stickiness' of the chromatin. The peripherally-retracted aggregates form the 'chromatinic rim' at this site. The remaining chromatin aggregates retract to form chromocentres, possibly around centromeres.

6. Hardening, which is probably dependent on nuclear protein composition, can arrest shrinkage and retraction at any stage of the above processes.

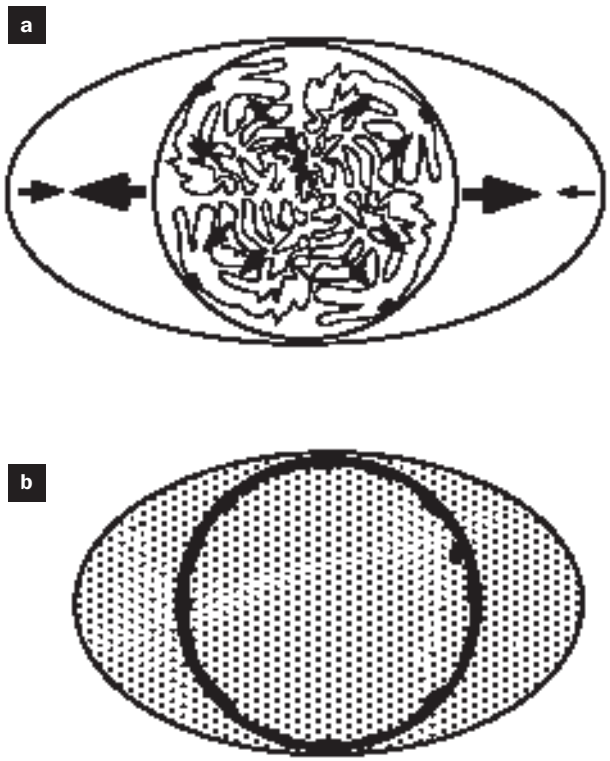
The timing and intensity of each of these processes depends on qualitative and quantitative aspects of the macromolecular structure of the various regions (perinuclear, peripheral nuclear and centronuclear) involved. If perinuclear cytoplasm is particularly susceptible to shrinkage, the nucleus may become enlarged by a pull outwards (see section dealing with papillary carcinoma of thyroid).

Secondary factors affecting chromatin pattern

Although the above hypothesis is considered to be the general mechanism behind the appearance of 'chromatin pattern' in histologically processed cells, some additional factors may be relevant in certain circumstances:

1. Leaching of DNA and protein from sections during processing. This has been extensively documented in the literature.[45] Up to 30% of DNA can be lost from sections during processing.[9] Fixatives (especially acidic ones[46]) that cause breakdown of nucleic acids or harden tissues only slowly might be associated with greater leaching. The qualities and quantities of the DNA-binding nuclear proteins also may be relevant.

Fig. 3. Formation of enlarged 'egg shell' ('orphan Annie') nuclei.



(a) Early fixation. Hardening of the nucleus is weak or delayed, and cytoplasmic shrinkage is strong and rapid. Therefore, cytoplasmic retraction draws the nuclear membrane outwards, and enlarges the nucleus.

(b) Complete fixation. As the nucleus is not hardened, retraction proceeds to the extreme, producing 'egg shell' chromatinic rim in an enlarged nucleus. Chromocentres, along with nucleoli, may be retracted to the periphery or possibly lost by leaching.

2. Native or artefactual chromosome-chromosome interactions. Although the existence of native chromosome-chromosome attachments are controversial, such sites might form centres for the collapse of chromatin, with formation of chromocentres, in addition to those forming by collapse onto central chromomeres.

3. Inhibition of 'complete' chromatin collapse by prior 'incomplete' collapse, due to bridging or air-drying. Bridging (aldehydes) or irregular collapse (air-drying) may inhibit the fullest shrinkage/collapse of chromatin by ethanol. This would explain how pure ethanol (as used in the Papanicolaou method) produces more sharply-defined chromatin aggregates in fresh/wet material than it does in either air-dried or formalin-fixed smears.

Discussion

The model offers an explanation for many common observations of histologically-processed nuclei.

'Coarse' versus 'fine' chromatin pattern
For a coarse pattern, shrinkage of aggregated chromatin might occur over a relatively long period of time before it is arrested by hardening. For a fine pattern, shrinkage of aggregated chromatin might occur over a relatively short period of time before it is arrested by hardening.

Effects of air drying on chromatin pattern
Air-drying is useful only in the preparation of cytological smears, as cells attach to the slide and spread before drying. This prevents the marked shrinkage that occurs by removal of water without replacement by another solvent (such as ethanol[8]). Such preparations, when stained without further fixation, show little chromatin pattern. When air-dried smears are immersed in methanol, however, a chromatin pattern appears. First, methanol partially re-expands the dried chromatin and then shrinks it, producing a limited form of the retraction process described above.

Different cell type appearances
As the major differences in nuclear composition between cell types are of nuclear sap proteins and RNA, it is proposed that the degree to which chromatin aggregation occurs depends on qualitative and quantitative aspects of these proteins and RNA. In addition, shrinkage to the periphery depends on a relatively large volume of nuclear sap so that the retracted aggregates can leave behind a central zone. This amount of nuclear sap depends on the metabolic activity of the cell, which varies from cell type to cell type.

Common changes to nuclear appearance after 'activation' (Fig. 2)
'Activation' involves increases in metabolic activity, RNA transcription and nuclear sap protein content. Nuclear sap volume increases and the shrunken chromatin aggregates have more surrounding nuclear volume in which to retract to the periphery.

Enlargement and central clearing of nuclei in papillary thyroid carcinoma (Fig. 3)
After histological processing of papillary thyroid carcinoma, nuclei are larger and have more pronounced central clearing than is demonstrable in corresponding frozen sections.[10] According to the current model, the perinuclear cytoplasm is more liable to shrinkage during fixation. Therefore, the cytoplasm, with the nuclear membrane attached, shrinks from the central zone of the nucleus. At the same time, chromatin aggregates and shrinks according to the model. Having an enlarged nuclear sap volume in which to shrink, the chromatin retracts fully to the periphery, causing the 'egg shell' appearance. For this to occur, the perinuclear cytoplasm and/or nuclear sap proteins and RNA must have different properties to those of follicular carcinoma, in which central nuclear clearing does not occur.

Light and electron microscopic appearances after aldehyde fixation
Although formaldehyde is often described as a poorer fixative than glutaraldehyde for electron microscopy, it may

not be so. Rather, it is likely that formaldehyde produces only partially shrunken chromatin aggregates (fuzzy edges on electron microscopy) and glutaraldehyde produces more intensely shrunken chromatin aggregates (sharper edges). Glutaraldehyde is known to produce almost total destruction of the α-helical protein structure.[9] This may be a mechanism by which glutaraldehyde produces more pronounced chromatin aggregation and shrinkage (either by itself or in response to subsequent dehydration, especially with acetone) than does formaldehyde.

Lack of chromatin pattern after osmium tetroxide fixation
Osmium tetroxide probably acts mainly through the oxidation of amino acids and other components, rather than by coagulation,[7-9] and there is evidence that it cross-links and cleaves proteins.[45] However, other mechanisms may be involved, such as interaction with carbonyl bonds.[9] In the present context, osmium tetroxide fixation first may disperse pre-existing chromatin aggregates by oxidation, and then, by not forming coagula, may fail to induce new chromatin aggregation. Although osmium tetroxide hardens and shrinks tissue,[47] these processes do not alter the dispersed state of the chromatin. Without significant aggregation, no chromatin pattern survives or develops.

Testing the hypothesis

The hypothesis outlined draws on many well-known observations of the effect of fixatives on the morphology of tissues, on the susceptibility of chromatin to non-chromosomal aggregation, and on more recent data derived from the use of biochemical and advanced microscopical techniques. The hypothesis is testable in various ways, using both long-established and recently developed research techniques.

Visualising the shrinkage and retraction of chromatin in living cells during fixation
An important step in testing the model involves documentation of the shrinkage and peripheral retraction of chromatin of appropriate cells during fixation with ethanol but not with osmium tetroxide. Baker[7] described some experiments of this type using tissue-cultured cells. Additionally, the effects of post-fixation agents, such as xylene, should be investigated.

Several forms of microscopy might be considered, although most are associated with technical difficulties. Ordinary light microscopy with supravital nuclear staining is an option but these stains bind to chromatin and may destabilise the dispersion of chromatin in the living nucleus and lead to unreliable results.

Time-lapse phase-contrast microscopy might be attempted. However, with this type of microscopy, it is difficult to distinguish events occurring in the nucleus from those in the underlying and overlying cytoplasm. Similarly, without staining of chromatin, events in nuclear vacuoles and other structures could provide misleading impressions.

Time-lapse confocal microscopy of living cells has been reported.[47-50] This form of microscopy permits the effect of structures above and below the nucleus to be excluded, and hence may be of considerable value in the assessment of chromatin aggregation.

Time-lapse fluorescence microscopy of nuclei stained with fluorescent anti-centromere antibodies to investigate whether or not centromeres change location during fixation is possible. Double staining with another chromosome site-specific probe[51] for telomeric chromatin has been used, and thus the relative repositioning of telomeric versus centromeric chromatin during fixation could be defined. However, the optimum equipment for studies of this kind would be the laser-scanning cytometer, which can provide information on multiple parameters in individual cells.[52]

Experimental **in vitro** *studies of shrinkage of simple proteins*
In vitro studies of the shrinkage of simple protein preparations[7,8] (such as serum albumin and albumin-gelatin gels) would test the effects of prior aldehyde and air-drying on shrinkage induced by ethanol. Use of protein preparations not exposed to prior denaturing-extractive processes, such as cold ethanol used in Cohn fractionation of plasma proteins and detergents used for the purification of membrane proteins, would be important.

Furthermore, as proteins in general are highly specific in their amino acid composition and high order structure, studies of protein preparations not found in nuclei might fail to yield relevant or conclusive results.

Leaching of chromatin and other nuclear contents
Most methods used to estimate leaching of DNA rely on Feulgen reaction-based estimates of DNA in nuclei, both before and after individual processing steps.[53] However, with more sensitive tests of DNA now available,[54] it might be more accurate to estimate DNA in the fixative and washing fluids after processing a nucleus-rich tissue such as the thymus. Tissues with no DNase activity would be appropriate.

Significance and conclusions

The present hypothesis addresses long-recognised observations of the different effects of histological processing on the appearances of cell nuclei, in terms of recent discoveries of the composition and internal structure of the nucleus. It explains the appearances observed by histopathologists and cytopathologists by reference to current cell biological and biochemical concepts.

It points to the physicochemical properties of nuclear non-histone proteins in general (rather than the restricted class of nuclear matrix proteins[55]) as the major factors in determining the patterns of chromatin in normal and pathological cells, and is an under-explored topic. If individual nuclear proteins could be cloned and purified in sufficient quantities without denaturation, then it might be possible to investigate interactions with chromatin under pathological conditions. The effect of small amounts of particular proteins micro-injected into single nuclei could be studied and those that significantly altered nuclear appearance, and to which antibodies could be raised, might be of diagnostic value in histopathology and cytology.

In studies of cancer cells, the nuclear non-histone protein composition may vary between well- and poorly-differentiated tumours, and the role of any specific nuclear non-histone protein discovered would be of prognostic value. Similarly, it is possible that documented schemata of nuclear non-histone proteins present in different tumours

would assist in classifying these lesions.

It is unlikely that alternative methods of microscopic examination will replace conventional fixation and paraffin-embedded sectioning in histopathology, for reasons of convenience, cost and the practical value of currently identified chromatin patterns. However, better understanding of the basis of nuclear chromatin patterns could lead to better histological processing practices and provide a sounder basis for the morphological findings in disease processes. □

The author is grateful to John Dore (principal hospital scientist and formerly laboratory manager, Division of Tissue Pathology, Institute of Medical and Veterinary Science) for discussions during the progress of this work, and for review of the manuscript.

References

1 Singer C, Underwood EA. *A short history of medicine.* 2nd edn. Oxford: Oxford University Press, 1962.

2 Virchow R. *Cellular pathology, as based upon physiological and pathological histology*. 2nd edn, 1858. (Translated by F. Chance) New York: Dover Publications Inc, 1971.

3 Ford CE. Pathological consequences of chromosomal abnormality. In: Florey HW, ed. *General pathology*. 4th edn. London: Lloyd-Luke, 1970: 590.

4 Puchtler H, Meloan SN. On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions. *Histochemistry* 1985; **82**: 201-4.

5 Maximow AA, Bloom W. *Textbook of histology*. 5th edn. Philadelphia: Saunders, 1948: 1-25.

6 Bibbo M, ed. *Comprehensive cytopathology* Philadelphia: Saunders, 1991: Ch 1 and 474.

7 Baker JR. *Principles of biological microtechnique. A study of fixation and dyeing* (1958). London: Methuen, reprinted 1970: 19-154.

8 Boon ME, Drijver JS. *Routine cytological and staining techniques*. London: MacMillan Education, 1986.

9 Bancroft JD, Stevens, A. *Theory and practice of histological techniques*. 4th edn. Edinburgh: Churchill Livingstone, 1996.

10 Rosai J, Carangui ML, DeLellis RA. *Tumors of the thyroid gland*. Atlas of Tumour Pathology (Third Series, Fascicle 5). Washington DC: Armed Forces Institute of Pathology, 1990: 72-4.

11 Ghadially FN. *Diagnostic electron microscopy of tumours*. 2nd edn. London: Butterworths, 1985: 261.

12 Fawcett DW. *The cell*. 2nd edn. Philadelphia: Saunders, 1981: 204-12.

13 Jackson V. Formaldehyde cross-linking for studying nucleosomal dynamics. *Methods* 1999; **17**: 125-39.

14 Menzel A, Unteregger G. Two-dimensional electrophoretic analysis of nuclear proteins from human tumors. *Electrophoresis* 1989; **10**: 554-62.

15 Cupo JF, Lidgard GP, Lichtman WF. A high resolution two-dimensional gel electrophoresis and silver staining protocol demonstrated with nuclear matrix proteins. *Electrophoresis* 1990; **11**: 500-4.

16 Cupo JF. Electrophoretic analysis of nuclear matrix proteins and the potential clinical applications. *J Chromatogr* 1991; **569**: 389-406.

17 Alberts B, Bray D, Lewis J *et al*. *Molecular biology of the cell* . New York: Garland Publishing Inc, 1989: 483-504.

18 Belmont AS, Dietzel S, Nye AC, Strukov YG, Tumbar T. Large-scale chromatin structure and function. *Curr Opin Cell Biol* 1999; **11**: 307-11.

19 Lewin B. *Genes VI* Oxford: Oxford University Press, 1997: 752-6.

20 Bradbury EM. Nucleosome and chromatin structures and functions. *J Cell Biochem Suppl* 1998; **30-31**: 177-84.

21 Qumsiyeh MB. Structure and function of the nucleus: anatomy and physiology of chromatin. *Cell Mol Life Sci* 1999; **55**: 1129-40.

22 Nicolini C, Carrara S, Mascetti G. High order DNA structure as inferred by optical fluorimetry and scanning calorimetry. *Mol Biol Rep* 1997; **4**: 235-46.

23 Mirsky AE, Osawa S. The interphase nucleus. In: Brachet J, Mirsky AE, eds. *The cell: biochemistry, physiology, morphology*. New York: Academic Press, 1961; 677-770.

24 Foe VE, Alberts BM. Reversible chromosome condensation induced in Drosophila embryos by anoxia: visualization of interphase nuclear organization. *J Cell Biol* 1985; **100**: 1623-36.

25 Milas L, Stephens LC, Meyn RE. Relation of apoptosis to cancer therapy. *In Vivo* 1994; **8**: 665-73.

26 Robertson JD, Orrenius S, Zhivotovsky B. Review: nuclear events in apoptosis. *J Strucutural Biol* 2000; **129**: 346-58.

27 Tang DG, Porter AT. Apoptosis: a current molecular analysis. *Pathol Oncol Res* 1996; **2**: 117-31.

28 Chambers R, Fell HB. Micro-operations on cells in tissue culture. *Proc Soc R Soc (Lond) Ser B* 1932; **B109**: 380-403.

29 Weiss L. *Histology, cell and tissue biology*. 4th edn. New York: Elsevier Science Publishing, 1983: 25.

30 Allfrey V. The isolation of subcellular components. In: Brachet J, Mirsky AE, eds. *The cell: biochemistry, physiology, morphology*. New York: Academic Press, 1961: 193-290.

31 Evans WH. Organelles and membranes of animal cells. In: Findlay JBC, Evans WH, eds. *Biological membranes, a practical approach*. Oxford: IRL Press, 1987: 21-2.

32 Haaf T, Schmid M. Chromosome topology in mammalian interphase nuclei. *Exp Cell Res* 1991; **192**: 325-32.

33 Santos SJ, Singh NP, Natarajan AT. Fluorescence *in situ* hybridization with comets. *Exp Cell Res* 1997; **232**: 407-11.

34 Albertson DG, Fishpool RM, Birchall PS. Fluorescence *in situ* hybridization for the detection of DNA and RNA. *Methods Cell Biol* 1995; **48**: 339-64.

35 Spector DL Macromolecular domains within the cell nucleus. *Ann Rev Cell Biol* 1993; **9**: 265-315.

36 Cremer T, Kreth G, Koester H *et al*. Chromosome territories, interchromatin domain compartment, and nuclear matrix: an integrated view of the functional nuclear architecture. *Crit Rev Eukaryot Gene Expr* 2000; **10**: 179-212.

37 Visser AE, Jaunin F, Fakan S, Aten JA. High resolution analysis of interphase chromosome domains. *J Cell Sci* 2000; **113**: 2585-93.

38 Stuurman N, Meijne AM, van der Pol AJ, de Jong L, van Driel R, van Renswoude J. The nuclear matrix from cells of different origin. Evidence for a common set of matrix proteins. *J Biol Chem* 1990; **265**: 5460-5.

39 Nelson WG, Pienta KJ, Barrack ER, Coffey DS. The role of the nuclear matrix in the organization and function of DNA. *Annu Rev Biophys Biophys Chem* 1986; **15**: 457-75.

40 Bosman FT. The nuclear matrix in pathology. *Virchows Arch* 1999; **435**: 391-9.

41 Pederson T. Half a century of 'the nuclear matrix'. *Mol Biol Cell* 2000; **11**: 799-805.

42 Gruenbaum Y, Wilson KL, Harel A, Goldberg M, Cohen M. Review: nuclear lamins – structural proteins with fundamental functions. *J Struct Biol* 2000; **129**: 313-23.

43 Stuurman N, Heins S, Aebi U. Nuclear lamins: their structure, assemby and interactions. *J Struct Biol* 1998; **122**: 42-66.

44 Hutchinson CJ, Alvares-Reyes M, Vaughabn OA. Lamins in disease: why do ubiquitous nuclear envelope proteins give rise

to tissue-specific disease phenotypes. *J Cell Sci* 2001; **114**: 9-19.
45 Hopwood D. Cell and tissue fixation 1972-82. *Histochem J* 1985; **17**: 389-442.
46 Koshiba M, Ogawa K, Hamazaki S, Sugiyama T, Ogawa O, Kitalima T. The effect of formalin fixation on DNA and the extraction of high-molecular-weight DNA from fixed and embedded tissues. *Pathol Res Pract* 1993; **89**: 66-72.
47 Wollweber L, Stracke R, Gothe U. The use of a simple method to avoid cell shrinkage during SEM preparation. *J Microsc* 1981; **121**: 185-9.
48 Bornfleth H, Edelmann P, Zink D, Cremer T, Cremer C. Quantitative motion analysis of subchromosomal foci in living cells using four-dimensional microscopy. *Biophys J* 1999; **77**: 2871-86.
49 White WM, Rajadhyaksha M, Gonzalez S, Fabian RL, Anderson RR. Non-invasive imaging of human oral mucosa *in vivo* by confocal reflectance microscopy. *Laryngoscope* 1999; **109**: 1709-17.
50 Manders EM, Kimura H, Cook PR. Direct imaging of DNA in living cells reveals the dynamics of chromosome formation. *J Cell Biol* 1999; **144**: 813-21.
51 Sullivan KF, Shelby RD. Using time-lapse confocal microscopy for analysis of centromere dynamics in human cells. *Methods Cell Biol* 1999; **58**: 183-202.
52 Nagele RG, Freeman T, McMorrow L, Thomson Z, Kitson-Wind K, Lee HY. Chromosomes exhibit preferential positioning in nuclei of quiescent human cells. *J Cell Sci* 1999; **112**: 525-35.
53 Darzynkiewicz Z, Bedner E, Li X, Gorczyca W, Melamed MR. Laser-scanning cytometry: a new instrumentation with many applications. *Exp Cell Res* 1999; **249**: 1-12.
54 Chieco P, Derenzini M. The Feulgen reaction 75 years on. *Histochem Cell Biol* 1999; **111**: 345-58.
55 Sambrook J, Fritsch EF, Maniatis T. Quantitation of DNA and RNA. In: *Molecular cloning.* 2nd edn. Cold Spring Harbour: Cold Spring Harbour Laboratory Press, 1989: E5-E7.
56 Hughes JH, Cohen MB. Nuclear matrix proteins and their potential applications to diagnostic pathology. *Am J Clin Pathol* 1999; **111**: 267-74.