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THE USE OF SCANNING ELECTRON MICROSCOPY FOR INVESTIGATIONS INTO THE THREE DIMENSIONAL ORGANISATION OF THE INTERPHASE NUCLEUS

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Abstract

In an attempt to elucidate three dimensional information on the organisation of the nucleus, nuclei have been extracted from ascites tumour cells or tissue culture cells by a variety of biochemical techniques, and prepared for high resolution scanning electron microscopy using an osmium-thiocarbo- hydrazide infiltration procedure which has previously proved successful for analysis of chromosome structure. Nuclei were prefixed with either Methanol-Acetic acid, glutaraldehyde or formaldehyde and then extracted by a variety of detergents with the aim of a 'biochemical dissection' of their outer elements to allow surface visualisation of the nuclear lamina. Continued extraction removed all aspects of the nuclear periphery and allowed direct visualisation of the in situ organisation of the chromatin, apparent as at least two levels of supercoiling.

Key Words: Scanning Electron Microscopy of Nuclei, Nuclear Membrane, Interphase Chromatin, Internal Nuclear Organisation.

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Introduction

Although conventional transmission microscopy (TEM) has produced a wealth of information regarding the structure of the cytoplasm and its organelles, the nucleus has remained relatively less well characterised. This is largely because of the difficulty of tracing a 30nm unit fibre of chromatin through the successive 200 serial thin sections which would be required to investigate a nucleus 10μm in diameter. As the chromatin at 30nm is already three fifths of the thickness of each section, and apparently runs a tortuous course, very little of the 3D fibre organisation within the nucleus has been elucidated to date.

The alternative approach, which has been very successful, in terms of generating images of both replicating and transcribing chromatin, has been to produce extracted chromatin preparations which are subsequently spread on a carbon support film and viewed after rotary shadowing in the TEM. This approach has produced the majority of our knowledge concerning the structure of chromatin (see Igo-Kemenes et al 1982 for review). Perhaps the most spectacular images are the 'Christmas tree' morphology of nucleolar transcription units of amphibian, insect (Miller and Beatty 1969) and mammalian cells (Harper and Puvion-Dutilleul 1979). In all these methods however, great care is taken to disperse and spread the chromatin preparation in an entirely two dimensional manner, for optimal transmission microscopy. Delightful as the appearance of such transcriptional units is, it is unlikely that the biological activity associated with these structures is undertaken in this two dimensional form within the interphase nucleus, and important three dimensional spatial relationships present in the intact nucleus are probably disrupted by this approach. Other biochemically isolated aspects of nuclear structure, such as the nuclear 'cage' (Cook and Brazell 1979) or matrix (Pienta and Coffey 1984) also have yet to be visualised in other than spread or sectioned preparations. Kirschner et al (1977) isolated whole nuclei from liver and exposed details of the nuclear envelope and pores after detergent extraction with Triton X-100. The fine structure of the pore complexes

was clearly visible with the high resolution microscopy employed, including 8 fold symmetry after Markham Rotation. However, when the nuclear membranes were removed, the peripheral chromatin was not investigated in any detail. The remainder of chromatin visualisation in the SEM has usually been initiated from metaphase chromosome preparations, although various stages of prophase organisation have also been reported (Gollin et al 1984; Mullinger and Johnson 1984). A type of freeze fracture study, involving delayed fixation and thawing, also allows three dimensional organisation in the interphase nucleus to be observed (Haggis and Bond 1979, Haggis et al 1983).

In order to investigate the possibility of SEM for the study of the three dimensional organisation of the nucleus, we have adapted methods from our own SEM studies of chromosome structure using an osmium-TCH infiltration to improve secondary electron emission, together with high efficiency collection in an 'in lens' electron optical system (Allen et al 1985, 1986, 1988).

Materials and Methods

Nuclear Isolation

A variety of methods for nuclear extraction were investigated, involving hypotonic buffers alone, a mixture of hypotonic buffer and detergents, or detergents alone. The hypotonic solutions were usually based on a Tris-HCl (10mM) buffer, adjusted to pH 7.4. In some cases homogenisation using a hand-held homogeniser was required, in other cases not. In all cases we found it best to monitor the effect of the hypotonic solutions using a phase contrast microscope, homogenising if the nuclei were not free of their cytoplasm after the first five to fifteen minutes exposure to hypotonic buffer. The cell types used were Yoshida ascites from rat, human lymphocytes from short-term culture (72 h) and Indian Muntjac fibroblasts from routine tissue culture. The best conditions for nuclear isolation were different for each cell line, and could be produced in alternate ways for the same line (e.g. buffer or buffer plus detergent). Occasionally cells would not rupture and release their nuclei, and contaminating cytoskeletal elements were apparent in the SEM. However, in general there was a highly uniform appearance to the extracted nuclei. Detailed methodology for the Yoshida ascites and Muntjac fibroblasts are given below.

Nuclear isolation procedure for Yoshida Ascites (O'Connor 1987)

Male Wistar rats were sacrificed 7 days after injection of 10^7 ascites cells. The ascites were harvested from the sacrificed animals by an intra-peritoneal injection of 24 mls heparanised phosphate buffered saline (PBS) which was withdrawn using a 20 ml syringe, containing the ascites cells which were briefly stored at 4°C, prior to nuclear isolation. The nuclei were isolated from the cells using two buffers, NB 1 and NB 2.

Note: Apparently incompatible magnifications between ascites nuclei (average diameter 3.0μm) and Muntjac nuclei (average diameter 10μm) are due to their large differences in size. ➡

Fig. 1. Ascites nucleus, isolated as per protocol fixed in glutaraldehyde and osmium-TCH infiltrated. The surface of the nucleus is largely granular with some fibrous elements (probably intermediate filaments). Field width = 6.0μm

Fig. 2. Detail of isolated Muntjac nucleus showing a largely granular substructure, possibly ribosomes retained at the surface of the nuclear membrane. Field width = 2.9μm

Fig. 3. Isolated ascites nucleus, retaining many cytoskeletal filaments at the surface. Field width = 4.0μm

Fig. 4. Ascites nucleus showing gross retention of cytoskeletal organisation, which appears as a 'cage' around the nucleus. Field width = 15μm

Fig. 5. Detail of insufficiently isolated cell, showing remnants of the smooth, and obviously fenestrated cell membrane, exposing the underlying cytoplasmic contents. Field width = 4.0μm

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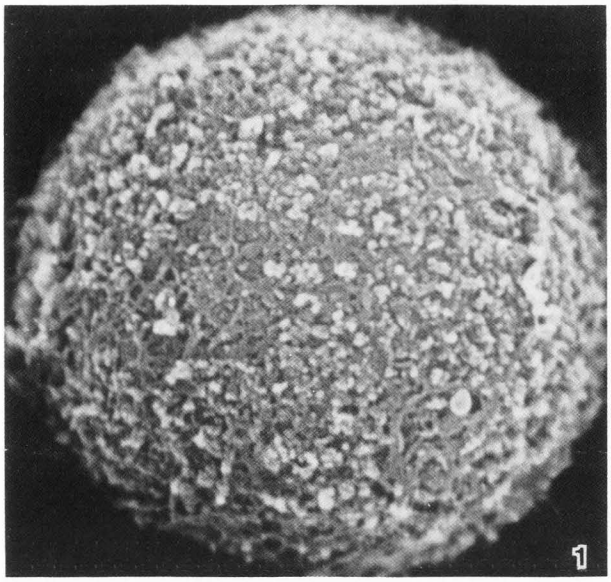
NB 1 320M sucrose
3mM $MgCl_2$
10mM Tris HCl
1mM Phenyl methyl sulphonyl fluoride (PMSF)
adjusted to PH 7.4
to which 0.35% (v/v) Triton X 100 was added

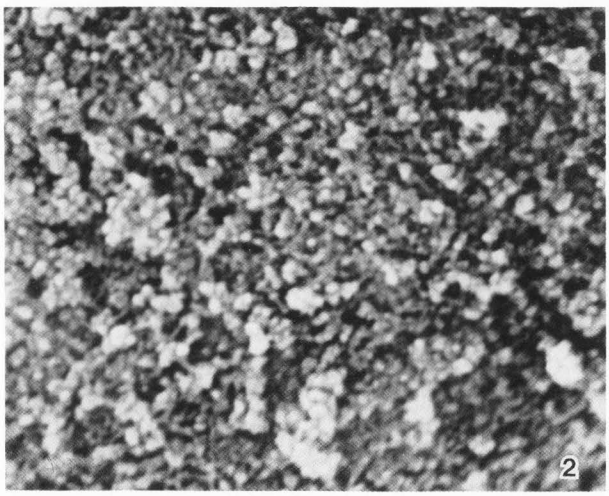
NB 2 as NB 1 but with
0.25 M sucrose and without Triton

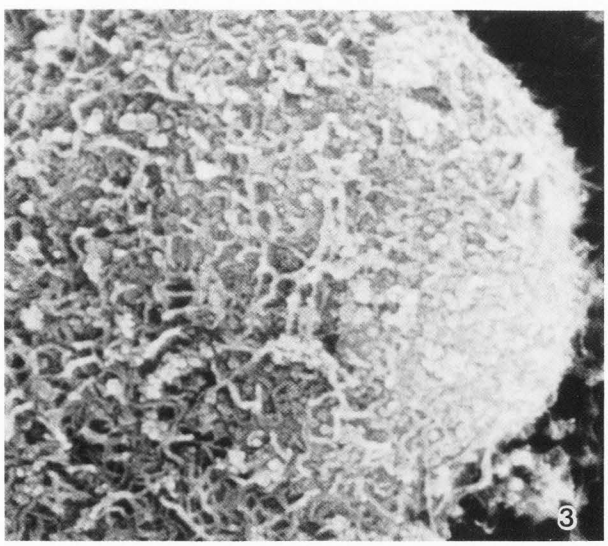
Cells were centrifuged for 5 mins (1000g) and resuspended in NB 1 without Triton for 3-5 min before homogenisation by hand in a glass homogeniser (25 strokes, 15-20 sec per stroke). The cells were then spun at 1000g for 15 mins, and resuspended in 50mls of NB 1 containing 0.35% Triton X 100 using a Pasteur pipette. The mixture was placed on a rotary stirrer for 30 min followed by centrifugation at 1000g for 10 min. The nuclei were washed once with NB 2, and resuspended ready for application to cover slips using a cytospin (see below).

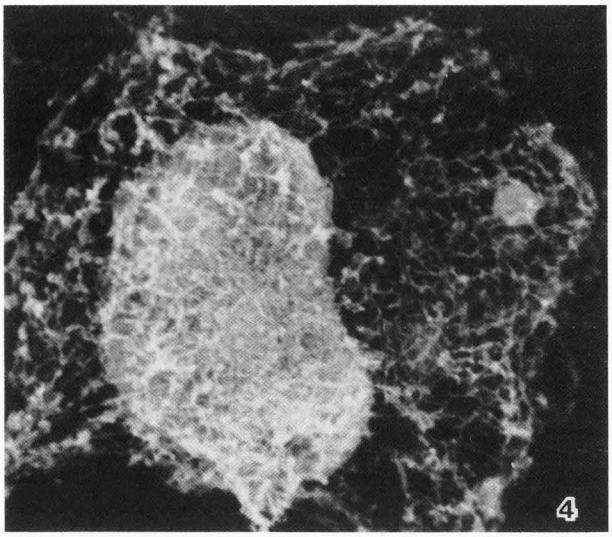
Isolation procedure for Muntjac nuclei

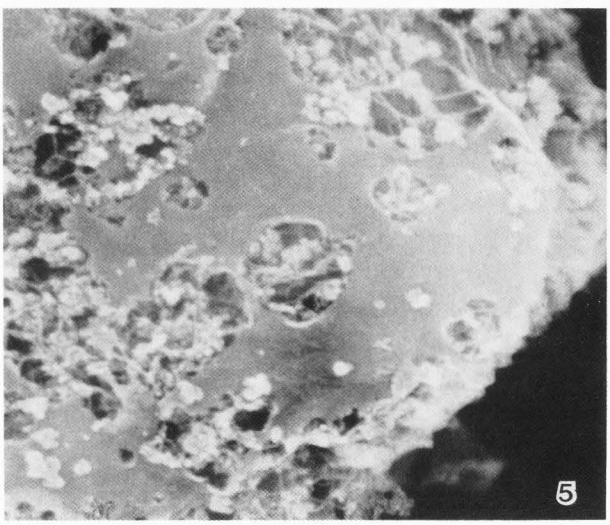
Muntjac cells were grown to confluence, rinsed with medium without serum and trypsinised (0.5%) for 10 min @ 37°C. The trypsin was quenched with serum, and the cells spun and resuspended in 75mM KCl, twice, adjusting the cell density to 2×10^5 cells/ml. The cells were left in KCl for 20 min at room temperature and then for 10 min on ice. At this stage, 1 ml of Triton X 100 was added to each 10 ml of cell solution, the centrifuge tube was shaken and left for a further 10 mins. No effort was made to dissolve all the Triton. One passage in and







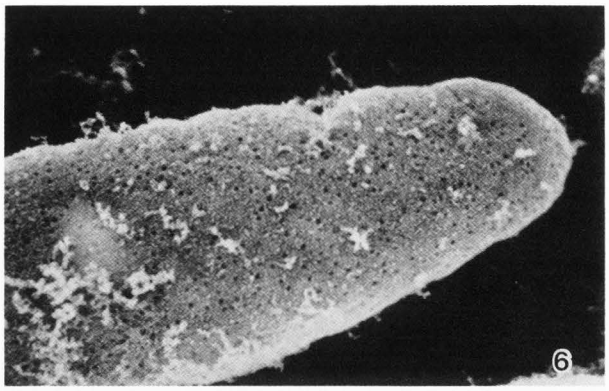


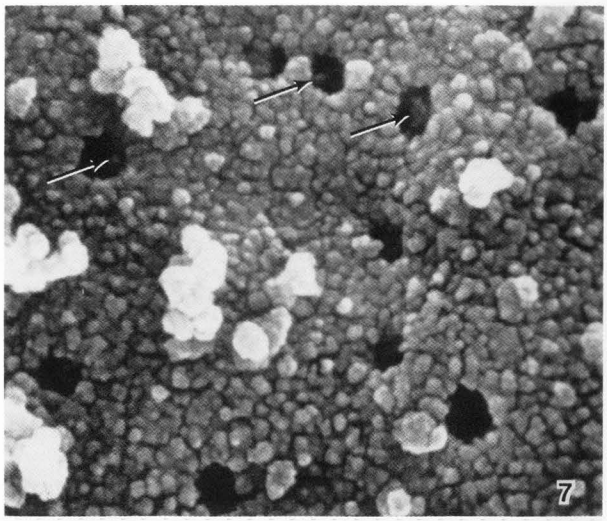


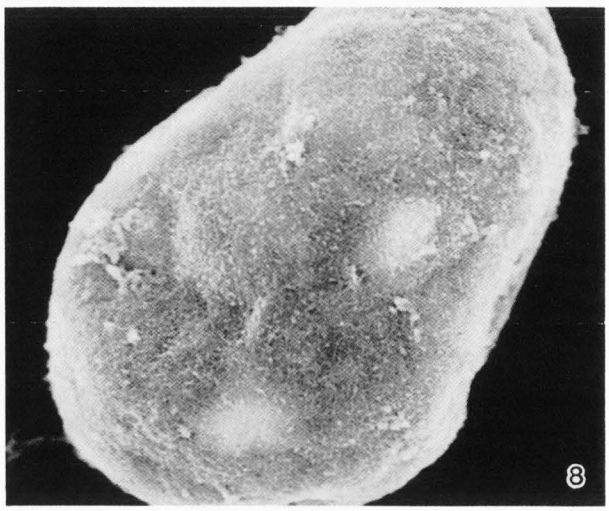
out of a 21 gauge needle was then sufficient to liberate the nuclei which were then cytospun onto 15mm coverslips. Alternatively, the cells could be placed directly in a sodium borate buffer (0.25mM pH 8.5) and left to swell and burst (5-10 min, 0°C) releasing the nuclei. A further alternative method was that of Seki et al (1987) which utilises 'Joy' (Procter and Gamble, USA) detergent to release and lyse chicken erythrocyte nuclei was also attempted. When used with Muntjac cells however 20 strokes with a homogeniser (in the isolating mixture) were required to liberate the nuclei from the cell membranes and cytoplasm.

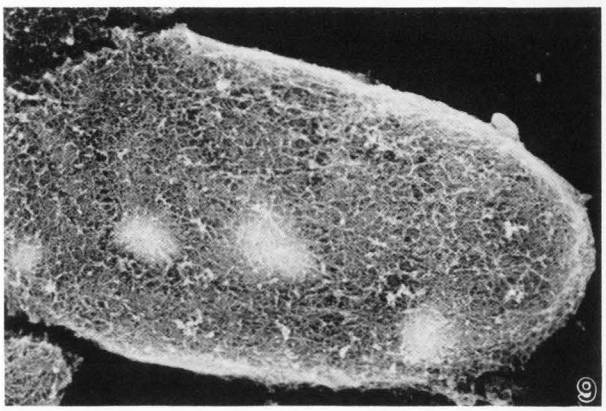
Deposition of Nuclei on coverslips

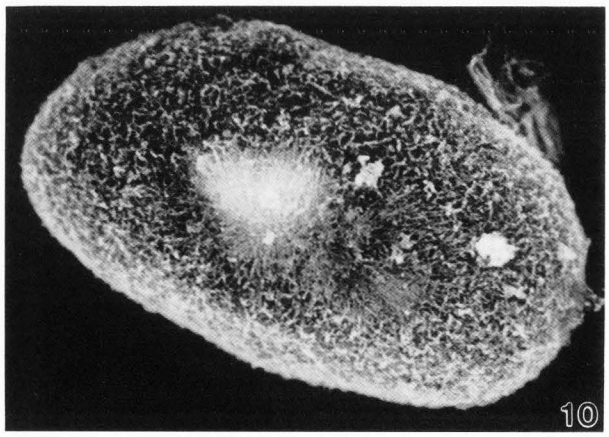
Nuclei were spun onto 15 mm coverslips using a 'cytospin' (Shandon Scientific UK) at the above concentration (2.5×10^5) for 3 min at 1000 rpm. The coverslip was placed on a slide under the funnel of the cytospin, and 400µl lit drop applied to the funnel. This amount was used to avoid air drying during spinning or during subsequent removal of the coverslips. In

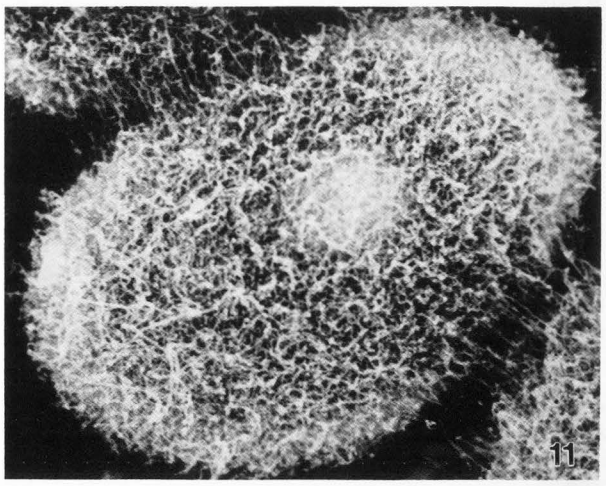












our hands, this protocol produced an evenly distributed monolayer of nuclei over a circle of 5mm diameter in the centre of the coverslip. The coverslips themselves were handled throughout the subsequent extraction or fixation procedures.

Detergent extraction of nuclei

After isolation, the nuclei were exposed to a variety of protocols involving the use of a variety of detergents, namely Sarkosyl NL35 (Sodium dodecyl sarcosinate, Serva Fine Chemicals, UK) SDS (Sodium dodecyl sulphate, BDH Chemicals, UK) or Joy (Procter and Gamble, Cincinnati, USA).

Fig. 6. Muntjac nucleus after isolation and 5 mins in double distilled water followed by 3.1 methanol-acetic prefixation, glutaraldehyde and osmium TCH. The surface of the nucleus appears relatively smooth, and nuclear pores are apparent. Field width = 10.5μm

Fig. 7. Detail of Fig. 6, (Hitachi S800 20kV). At high resolution there is a fine granular surface morphology between the nuclear pores, some of which show retention of granules (arrowed). Field width = 0.92μm

Figs. 8-11. Isolated Muntjac nuclei, showing successive loss of nuclear membrane with increasing time in 0.4% Sarkosyl after 3.1 methanol-acetic fixation. Fig. 8 5 mins Sarkosyl, Fig. 9 10 mins, Fig. 10 20 mins, Fig. 11 45 mins. All nuclei show particularly strong signal from the nucleolar regions. Field widths 8 = 9.0μm 9 = 9.5μm 10 = 17.5μm 11 = 21.0μm

Nuclei exposed directly to detergents very rapidly lost their integrity, so prefixation involving 3.1 Methanol: Acetic Acid, paraformaldehyde and glutaraldehyde were employed (see Results). After detergent extraction, the nuclei were refixed as follows.

Fixation

After isolation, the coverslips with nuclei were either fixed or subjected to other protocols (see below). At the end of the experimental procedures, or directly in the case of controls, all coverslips were prepared in the following protocol (all steps at room temp):

1. 3% Glutaraldehyde in M/15 Sorenson's phosphate buffer pH 7.2, 30 min -
2. 3 x buffer washes 5 min each
3. 1% Osmium tetroxide (OsO_4) in buffer 15 min
4. 2 x rinse in double distilled water (DDW)
5. Saturated solution Thiocarbohydrazide (TCH) (freshly prepared) 20 min
6. 2 x rinse DDW
7. 1% OsO_4 15 min
8. 2 x rinse DDW
9. TCH 20 min
10. 2 x rinse DDW
11. 1% OsO_4 15 min

Dehydrate through ethanol, critical point dry from liquid CO_2 using Freon 113 as intermediate solvent. View uncoated, or coat briefly using 2nm gold (sputter coating) or 2nm platinum deposited by ion beam etching or fast atom bombardment (Ion Tech Ltd. Teddington UK).

Specimens were observed at a variety of accelerating voltages on different instruments, either an ISI SS 40, operated at 20kV accelerating voltage or an ISI DS 130C or Hitachi S800 Field Emission microscope. Where the micrographs are from other than the ISI SS 40 instrument, this will be stated in the legend, together with operating conditions.

Results

Appearance of Extracted Nuclei

Almost regardless of the method of isolation, nuclei viewed in the SEM presented a consistent appearance. The granular surface morphology was interpreted to represent a thin layer of contaminating cytoplasm on the outer element of the nuclear membrane possibly composed mainly of ribosomes (Figs. 1, 2).

Occasionally a fibrous component was also apparent, consistent with the retention of perinuclear intermediate filaments (Fig. 3). Any gross cytoplasmic contamination, to the point where a 'network' of cytoskeletal filaments was retained, or areas of entire cell and cytoplasm with cell membrane were easily identified (Figs. 4, 5).

Visualisation of Nuclear membrane

It is apparent from the preceding observations that none of the variety of biochemical isolation techniques employed produced 'clean' separation between the cytoplasm and the outer element of the nuclear membrane. We found that the most effective way of removing the contaminating cytoplasm from extracted nuclei was a long exposure to isolating buffer (8 h in NB 2, 4°C) or, more conveniently, a brief rinse in double distilled water (4 min at 4°C) which produced typically smooth surfaced nuclei, with characteristic nuclear pores (Fig. 6). Pore size was in the normal range of 40 - 90nm, and high resolution microscopy revealed a granular surface morphology of the nucleus in these preparations, possibly indicative of some retention of cytoplasmic material. The majority of the pores did not show any internal structure, although some granules were occasionally apparent within the pores (Fig. 7). Further investigations to refine this technique are in progress, as these studies have not so far revealed the same level of information for nuclear pores as previously shown by Kirschner et al (1977).

Sub-membranous structures and Peripheral Chromatin

Having isolated nuclei as above, the aim was subsequently to expose sub-nuclear membrane organisation (the nuclear lamina) in situ against the peripheral chromatin, using detergent extraction of peripheral nuclear structure.

Use of all these detergents directly on isolated nuclei leads to rapid dissociation of nuclear integrity, so we have investigated several prefixations prior to detergent extraction. Fixatives included 3.1 methanol acetic acid, paraformaldehyde, glutaraldehyde and a mixture of paraformaldehyde and glutaraldehyde. Methanol-acetic acid fixation, although mainly considered to be a light microscope fixative, is used routinely as a prefixation technique in our chromosome studies, largely as it is the only preparative milieu to allow chromosomal spreads to be made. In terms of biochemical extraction, the work of Burkholder and Duczek (1982) has shown that Methanol-acetic acid is a highly retentive efficient fixative, removing a small amount of

histone H1, but little else. Paraformaldehyde was selected as it forms cross linkages between nucleic acids and proteins, whereas glutaraldehyde forms cross linkages between proteins alone (Sewell et al 1984). For a fuller consideration of methanol-acetic acid as a fixative in ultrastructural protocols see Allen at al (1988).

Both paraformaldehyde and glutaraldehyde were highly efficient in stabilising nuclear structure against the effects of extraction by detergents even when used at relatively low concentrations for short time periods (0.5% for 5 mins). In contrast, methanol-acetic acid produced a stabilisation of structure which allowed sequential removal of structure over reasonable periods of time (within 1 h) with Sarkosyl, SDS and Joy detergents. However, if material prefixed with either glutaraldehyde or paraformaldehyde or a mixture of both was exposed to similar detergent concentrations over longer periods (24 h), there was remarkable similarity between the two preparations.

Using sequential detergent extraction, a succession of morphologies of the nuclear periphery, peripheral chromatin itself and the subsequent dispersion of the entire nuclear chromatin could be produced. Brief extraction (5 mins Sarkosyl, Fig. 8) appeared only to begin to clear the surface of the outer nuclear membrane of cytoplasmic contamination (c.f. Fig. 1). However, successive increases in detergent exposure (10 mins, Fig. 9; 20 mins, Fig. 10) displayed an apparently sub-membranous and close packed feltwork (Fig. 9) at the nuclear surface which was successively lost with longer detergent extractions (Fig. 10) until the peripheral chormatin was readily apparent as dispersed fibres on the nuclear surface (45 mins detergent, Fig. 11). Whether or not the lamina has been optimally exposed by these particular extractions is uncertain, but as we can start with the entire nuclear periphery (Fig. 8) and finish with dispersed peripheral chromatin (Fig. 11) the potential for biochemical dissection of this structure would appear to be established. As the nuclear lamina occurs directly sub-adjacent to the nuclear membrane (Gerace et al, 1984) and can be reasonably expected to function as an interface, it is important that it should be visualised as a surface, rather than a profile as in TEM thin sections. One further striking feature in all these preparations is the amount of signal from the nucleolar region, even though it appears to be deep within the nucleus (Figs. 8-11).

Dispersed Chromatin

All external aspects of the nuclear membrane and lamina were removed by detergent treatment, exposing the nuclear chromatin. Extraction with detergents works more quickly (1-2 h) on unfixed isolated nuclei, and produces a dispersion of the chromatin, although individual nuclei are still apparent (Fig. 12). Prefixed nuclei (3.1 methanol-acetic acid) required longer (up to 24 h), to reach the same stage. The three dimensional organisation of the chromatin in these preparations is readily apparent (Fig. 13), particularly when observed in a high resolution instrument, where supercoiling of chromatin is resolved (Fig. 14). Clearly discernible variations in chromatin fibre diameter, indicative of the different levels of DNA packaging, are also observed (Fig. 15). Fibre diameters of 10nm, 25-35nm and 40-50 nanometers have been observed in the same field. These have been interpreted, respectively, as a fibre of single nucleosome diameter (possibly stretched chromatin) the typical unit fibre of chromatin, and further orders of chromatin supercoiling. These thickest fibres are often resolved to display a coiled sub-fibre organisation (Fig. 14). It is unlikely that some of the fibres represented cytoplasmic elements, as these were efficiently removed by the detergent treatment. However, other nuclear elements such as ribonucleoprotein, may also be present (Allen 1989).

The Internal Organisation of the Nucleus

The above methods allow a successively dispersed 'dissection' of nuclear components, but no access to the internal structure of intact nuclei. We have recently investigated this in two ways, firstly using a simple dry fracture technique, and secondly using a fast atom bombardment technique.

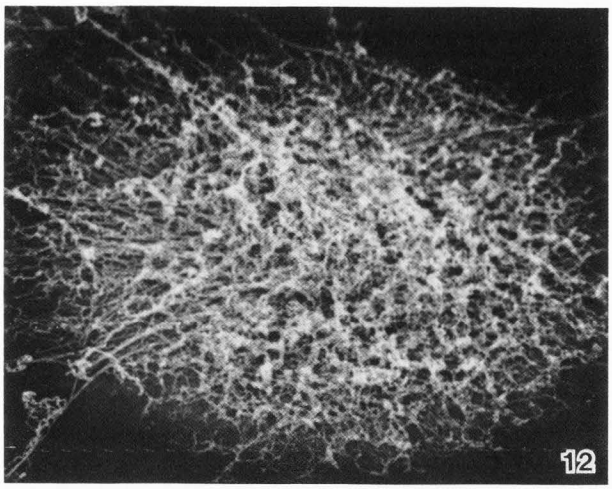
Dry Fracture

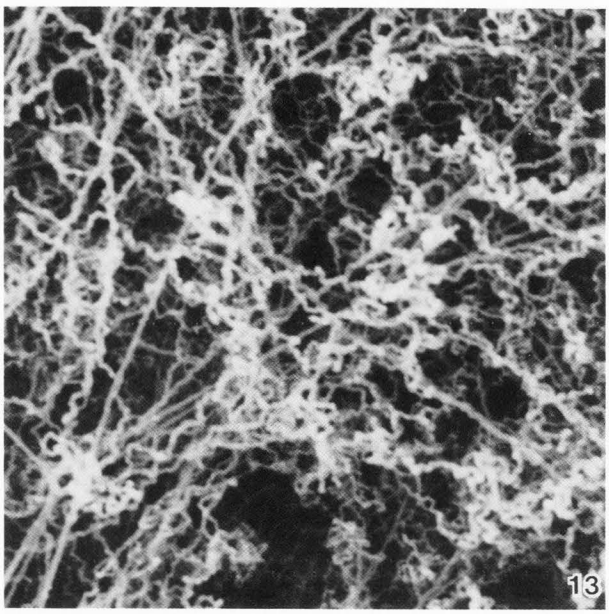
The dry fracture technique arose as the result of a chance observation of a nucleus which had become fractured during normal handling, thus indicating the brittle nature of Os-TCH infiltrated critical point dried nuclei. In view of this, deliberate fractures were produced by the simple means of bringing the coverslip and the nuclei [stuck to a Cambridge stub using an Adhesive Tab (Agar Aids UK)] together with another clean coverslip, also on a stub but with an adhesive tab on the upper side of the cover slip as well. The stubs were gently apposed, without lateral movement, and separated in a single straight clean pull. The adhesion of the nuclei to the coverslip to which they had been cytospun was sufficient to retain approximately half the nucleus, whereas the other half was removed by the adhesive tab. Complimentary fractures are in theory possible by this technique, but difficult to view as the adhesive itself is relatively unstable in the electron beam, even after coating.

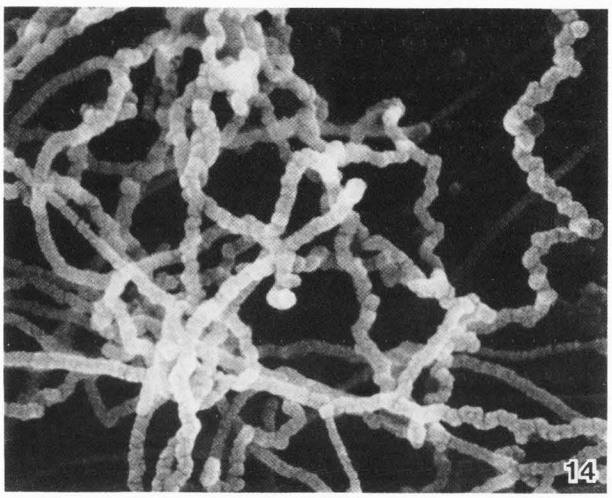
The results of this technique were encouraging, as the fracture surfaces appeared clean, and without distortion. The plane of fracture was quite variable, resulting in different aspects of the nucleus being exposed (Figs. 16, 17). More detailed examination revealed a consistency of appearance in similar fractures, and areas of denser packaging of chromatin on the inside of the nuclear membrane (Fig. 18) which were possibly consistent with the heterochromatic regions visualised in TEM sections. Individual chromatin fibres were readily resolved in these preparations.

Fast Atom Bombardment

The second method used to expose internal nuclear organisation was that of fast atom bombardment. It has already been shown that this technique does not suffer from the 'jagged mountain artefact' inherent in charged particle







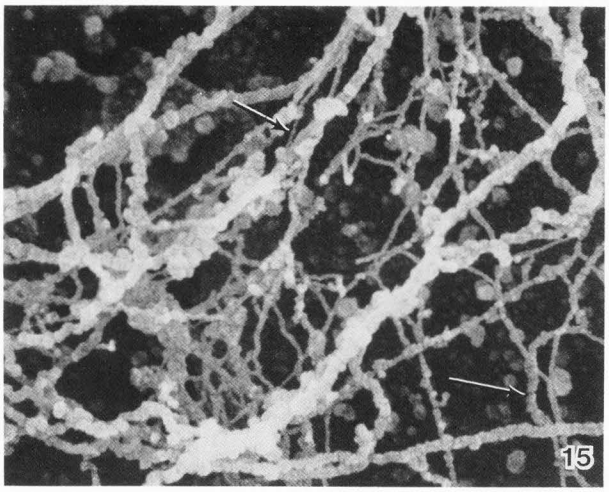


Fig. 12. Isolated nucleus, prefixed with 3.1 methanol-acetic, after 1 h exposure to 0.4% Sarkosyl, followed by glutaraldehyde, osmium TCH. The overall morphology of the nucleus has been lost, and the chromatin fibres are now more dispersed. Field width = 17.5μm

Fig. 13. Detail from nucleus in Fig. 12. The majority of chromatin fibres display a coiled or spiral appearance, although some rectilinear fibres run for long distances across the micrograph. Field width = 5.25μm

Fig. 14. Detail from Fig. 13, observed in ISI DS 130 top stage at 20kV. Most of the chromatin fibres appear supercoiled with diameters in the region of 50nms. Field width 2.0μm

Fig. 15. Detail of different region of the same preparation as Figs. 12-14 (also in ISI DSI 130 top stage). In this micrograph there is a clear variation in the diameter of chromatin fibres, ranging from 10nms (arrowed) to 40nms (arrowed). Field width = 2.0μm

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ion beam etching, and has been used to expose internal chromosome structure (Allen et al 1985, 1986). An Ion Tech (Teddington, UK) B365 EM microworkshop was used, at operating conditions of 4.5 mA at 4.5kV to etch nuclei from a low angle (7.5°) in order to minimalise residual damage at the surface. This apparatus also allows a platinum coating to be applied to the etched specimen without breaking the vacuum. As the specimens were already osmium-TCH infiltrated, coating was kept to approximately 1 nm in thickness. The extracted nuclei were etched for periods of 5 min at low angle. This is demonstrated in Fig. 19, in which a whole cell (prepared in the normal manner) which had survived the nuclear isolation procedure intact, is a good example of the action of fast atom bombardment action in etching off the surface material. The nucleus is clearly apparent amongst the cytoplasm, as are the two nucleoli,

which generate a very strong signal. Higher magnification of a similarly exposed nucleolus on the same stub reveals chromatin fibres and the largely fibrous structure of the nucleolus (Fig. 20). The lack of obvious residual damage at the plane of surface itself indicates the potential for exposing internal nuclear organisation with this technique. Successive etchings of the same area could lead to a 3 dimensional 'serial sectioning' technique which could be of great value in understanding overall nuclear organisation.

Discussion

In this overview, we have presented methods, illustrated with current results, which may be useful in the study of 3D nuclear organisation. Previous approaches have been largely two dimensional, either in the form of thin sections or spread preparations. Considering the importance of the processes which occur in the nucleus it is surprising that up to the present time, very little attention has been given to nuclear morphology in the SEM. Modern microscopes are well able to resolve down to the 10nm level, and preliminary observations (Allen unpub) have shown that this technology is perfectly compatible with immunocytochemical labelling of specific molecules using antibody-gold markers. A well established biochemical literature already exists for precisely characterised isolation procedures for nuclear matrix, nuclear envelope, nuclear lamins and the nucleolus itself, and as none of these has been visualised in three dimensions, this methodology remains unexploited by SEM. Furthermore, if the nuclei are isolated and then spun onto coverslips as above, the 100,000 nuclei per coverslip provide ample material for gel electrophoresis of extracted material directly from the specimen which is subsequently visualised in the SEM, bringing biochemistry and scanning microscopy together in as direct a way as possible.

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References

Allen TD, Jack EM, Harrison CJ, Claugher D. (1986) Scanning electron microscopy of human metaphase chromosomes. Scanning Electron Microsc. 1986; I: 301-311.

Allen TD, Jack EM, Harrison CJ, Claugher D, Harris R. (1985) Human metaphase chromosome preparation for scanning electron microscopy, a consideration of inherent problems. Science of Biological Specimen Preparation, 4th Pfefferkorn Conference. Becker, R. (Ed) Pub. SEM Inc. Chicago, IL. 299-314

Fig. 16. Isolated Muntjac nucleus after 3.1 methanol acetic prefixation, 5 mins Sarkosyl extraction, glutaraldehyde, osmium TCH infiltration, and dry fracture after critical point drying. The nucleus has been fractured revealing internal contents, within a shell like configuration of peripheral material presumably nuclear lamina and condensed chromatin, which is consistent with the thin layer of heterochromatin on the inner aspect of the nuclear membrane as seen in TEM sections. One nucleolus is apparent within the nucleus, although masked by chromatin fibres. Field width = 17μm

Fig. 17. Low power micrograph of nuclei after a dry fracture which removed the majority of the nucleus, leaving behind the substratum attached material, exposed on the inner aspect of the attached nuclear membrane. Field width = 35μm

Fig. 18. Detail of Fig. 17, showing individual chromatin fibres and a regular pattern of aggregation, possibly consistent with heterochromatin regions. Field width = 7.5μm

Fig. 19. Low power micrograph of whole cell after 5 mins fast atom bombardment at 7½°. The nucleus and nucleoli stand out clearly from the cytoplasm, after removal of approximately the upper half of the cell. Field width = 23μm

Fig. 20. Detail from same prep as Fig. 19, viewed in ISI DS 130 upper stage. Individual chromatin fibres are apparent, and the central nucleolus also appears fibrillar. No gross residual damage from the fast atom bombardment is apparent. Field width = 5.1μm

Allen TD, Jack EM, Harrison CJ. (1988) The three dimensional structure of human metaphase chromosomes as determined by scanning electron microscopy. In: Chromosomes and Chromatin, Vol II, (Ed) Adolph KW, CRC Press, Florida, USA, 1988.

Allen TD. (1989) Techniques for the study of the three dimensional organisation of chromatin in the interphase nucleus and metaphase chromosomes by SEM. In: Science of Biological Specimen Perparation, 7th Pfefferkorn Conference. In Press.

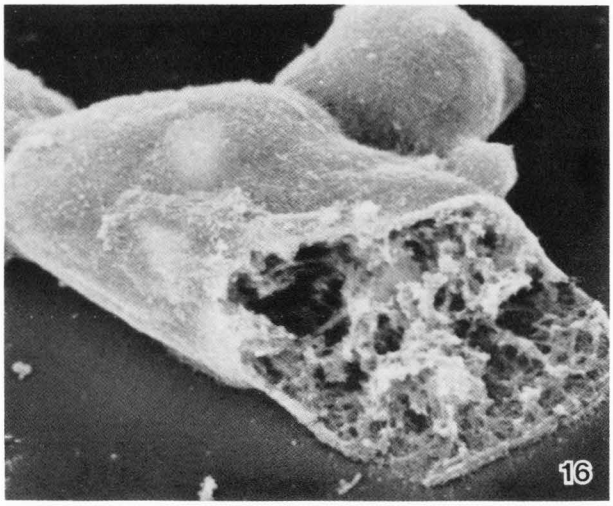
Burkholder GD, Duczek L. (1982) The effect of chromosome banding techniques on the histone and non-histone proteins of isolated chromatin. Can. J. Biochem. 60: 328-341

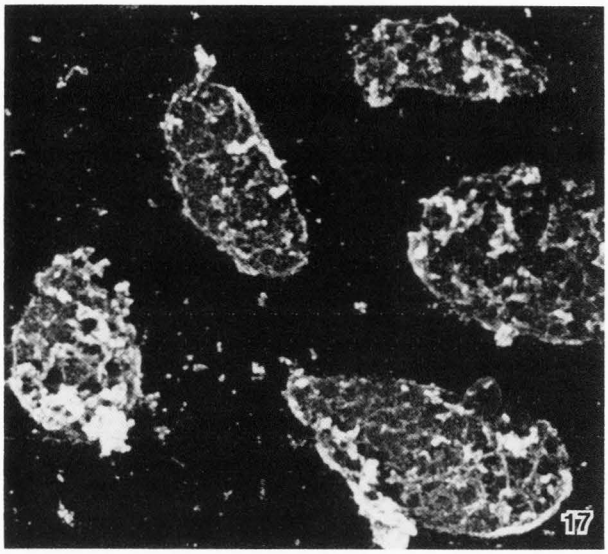
Cook PR, Brazell IA. (1979) Supercoils in Human DNA. J. Cell Sci. 19: 261-279

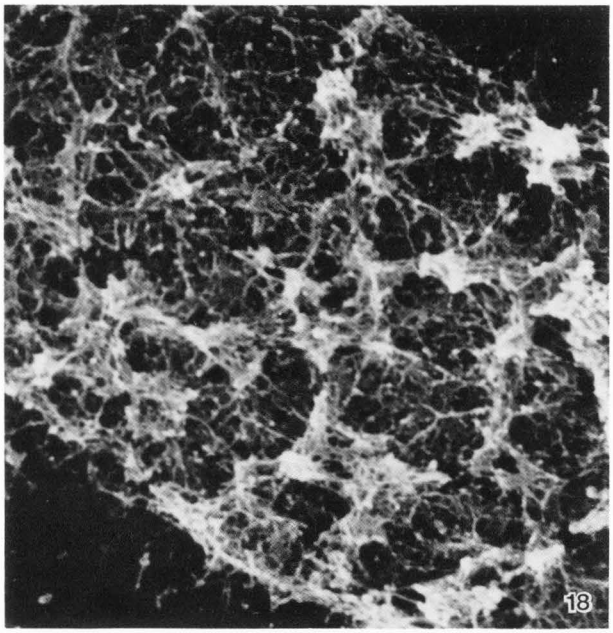
Gerace L, Comeau C, Benson M. (1984) Organisation and modulation of nuclear lamina structure. J. Cell Sci. Suppl. 1: 137-160

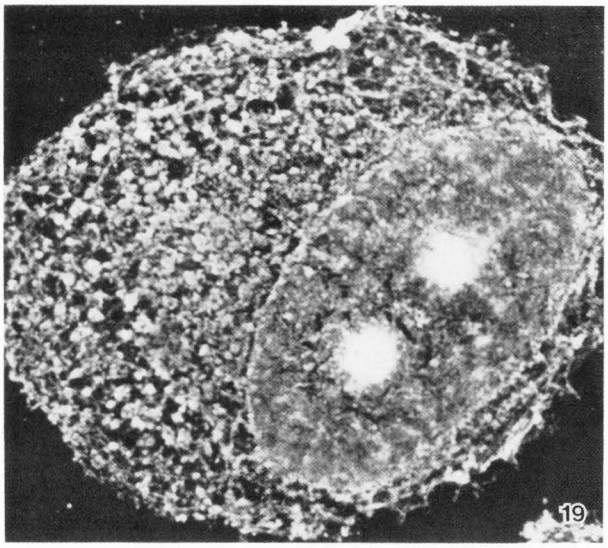
Gollin SM, Wray W, Hanks SK, Hittleman WN, Roa PN. (1984) The ultrastructural organisation of prematurely condensed chromosomes. J. Cell Sci. Suppl. 1: 203-210

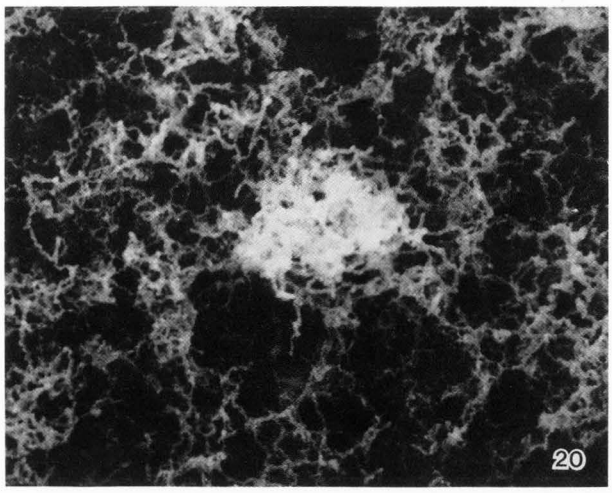
Haggis GA, Bond EF. (1979) Three dimensional view of chromatin in freeze fractured chicken erythrocyte nuclei. J. Microscopy, 115: 225-234











Haggis GH, Schweitzer I, Hall R, Bladon T. (1983) Freeze fracture through the cytoskeleton nucleus and nuclear matrix of lymphocytes studied by scanning electron microscopy. J. Microscopy 132: 185-194

Harper F, Puvion-Dutilleul F. (1979) Non-nucleolar transcription complexes of rat liver as revealed by spreading isolated nuclei. J. Cell Sci. 40, 181, 192

Igo-Kemenes T, Horz W, Zachan HG. (1982) Chromatin. Ann. Rev. Biochem. 51, 89, 121

Kirschner RH, Rusli M, Martin TE. (1977) Characterisation of the nuclear envelope, pore complexed and dense lamina of mouse liver nuclei by high resolution SEM. J. Cell Biol, 72: 118-132

Miller OL, Beatty BR. (1969) Visualisation of nucleolar genes. Science 164: 955-957

Mullinger AM, Johnson RT. (1984) Units of chromosome replication and packing. J. Cell Sci. 64, 179-192

O'Connor PM. (1987) Crosslinking reactions of the Dimethane sulphonates within chromatin. PhD Thesis, University of Manchester.

Pienta KJ, Coffey DS. (1984). A structural analysis of the role of the nuclear matrix and DNA loops in the organisation of the nucleus and chromosome. J. Cell Sci. Supp. 1: 123-137.

Seki S, Nakamura T, Suma F, Murakami M, Mori S, Oda T. (1987). Chromatin organisation in detergent lysed chicken erythrocyte nuclei. J. Electron Microscopy Technique 7: 223-231.

Sewell BT, Bouloukos C, Von Holt C. (1984) Fixation of Chromatin for electron microscopy. J. Microscopy 136: 103-112.

Discussion with Reviewers

K.W. Adolph: Is it possible to draw any conclusions from SEMs such as those in Figs. 1-4 regarding the mode of association of intermediate filaments with the nuclear envelope? Are specific attachments suggested, or are the intermediate filaments collapsed upon the exterior of the nucleus during isolation?
Authors: We think that at this stage, the suggestion of collapse of intermediate filaments on to the exterior of the nucleus is probably but the most likely explanation, but retention by attachment at the nuclear surface (see Figs. 21-22). However, there are specific methods for cytoskeletal isolation using known isolation buffers and detergents which may well be modified to retain the potential connections between nuclear elements and intermediate filaments. Our own preliminary studies show that when spherical cells in suspension are subjected to this type of extraction, the nucleus is often retained.

K.W. Adolph: How would the authors characterise the distribution of nuclear pores as in Figs. 6 and 7? Are nuclear pores evenly distributed, or are they found in patches? Do all nuclei from the same cells have the same density of pores? Are there differences in pore structure and distribution with nuclei of other cell types?
Authors: Our interest in nuclear pores to date has largely been confined to their appearance as an obvious 'signpost' of the interspace between cytoplasm and nuclear periphery. In a previous SEM study of nuclear pores, Kirshner et al (1977) investigated these parameters in some detail.

K.W. Adolph: Do the authors believe the variations in the diameters of the chromatin fibres in Figs. 13-15 represent different classes of fibres in the nucleus, or are they produced by stretching of the wider fibres?
Authors: We believe that the variations in diameter of chromatin do actually represent different classes of fibre within the nucleus, namely: a) mononucleosomal chromatin (approx 10-20 nms in diameter), b) A typical 'unit fibre' chromatin of 30-40 nms diameter, and c) supercoiled chromatin fibres of 60 nms diameter. Any intermediates between these classes probably arise by tension of the pre-existing fibres, as other elements such as cytoskeleton are efficiently removed by the detergent treatments.

K.W. Adolph: Does the chromatin that is adjacent to the inner nuclear envelope (as seen, for example, in Fig. 16) have any different structural properties than the more internal chromatin? Can anything be said about the nature of the attachment of chromatin fibres to the inner nuclear envelope?
Authors: At this relatively preliminary stage, with the nuclear dry fractures viewed with an instrument of modest resolution, we are only able to ascertain a 'shell' of peripheral chromatin, probably complexed with nuclear lamina proteins. However, with higher resolution, we would hope that with the ability to examine this peripheral chromatin 'in situ' that any alterations in the nature of chromatin in this situation relative to 'internal' chromatin, may become apparent. Recent studies (Allen 1989) have shown that this type of approach is compatible with gold labelling techniques, so that specific probes to the nuclear lamins for instance, can be used.

Reviewer II: Many of these treatments are very harsh (e.g. acid fixation, detergent extractions [SDS]) and without a parallel study it is difficult to know exactly what one is looking at after a specific treatment. For instance, Figs. 1 and 2 are interpreted to represent contaminating cytoplasm on the outer element of the nuclear membrane, but without the parallel thin-sections, this interpretation is speculative. It is my understanding that Triton X-100, used in the preparation of the nuclei, commonly strips off the outer membrane of the nuclear envelope, so these micrographs may actually be views of the outer side of the inner membrane.
Authors: We are currently investigating some of the treatments using sectioned pellets of nuclei in the TEM, which show that the surface of freshly isolated nuclei which appear 'clean' in the light microscope, do have surface contamination in the form of ribosomes and other cytoplasmic components as shown previously by Kirschner et al (1977) (Figs. 21-22). With this starting point, it seems reasonable to suggest that with increasing length of treatments known to remove these elements, it may be reasonably expected to successively remove material from the surface, and eventually reach the nuclear contents (i.e., chromatin), which does appear to be the case. Our proposal is that if this process can be demonstrated, as we believe we have, then the right length of extraction in the right buffer and detergent could well lead to the exposure of specific substructures in the nuclear periphery. What we were attempting to demonstrate in this initial series of experiments, was that the morphology of biochemical extraction, using conventional biochemical protocols considered acceptable for biochemical analysis, could be usefully observed using high resolution SEM.

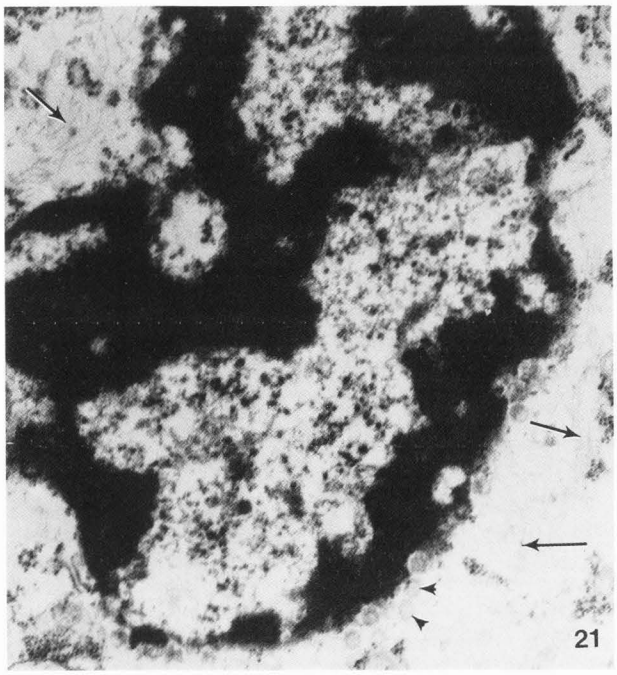


Fig. 21. Ascites nuclei fixed for TEM directly after separation from the remainder of the cell by hypotonic buffer. Both fine filaments (arrowed) and nuclear pore profiles (arrowheads) are apparent at the surface of the nucleus, which also displays residual ribosomes. Field width = 45 μm.

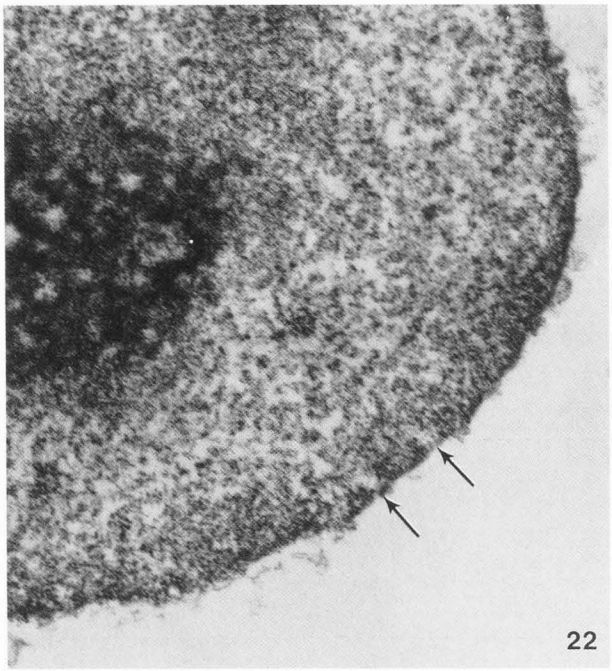


Fig. 22. Ascites nuclear profile after overnight storage and washing in nuclear isolation buffer. The surface membranes of the nucleus are no longer apparent, although channels in the peripheral chromatin (arrowed) may well indicate the original position of nuclear pores. Field width = 3.0 μm.

Reviewer II: In Fig. 6, following the double distilled water treatment, the authors claim that they are looking at the outer surface of the nuclear membrane. In fact, they could be looking at a water-induced variation of the inner membrane or possibly the underlying nuclear lamina or condensed chromatin with channels leading to the pore complex. The absence of annular granules around the "holes" makes me suspicious that this is not the outer surface of the nuclear envelope, unless the annular granules have been washed away.

Authors: We agree that double distilled water (albeit for 10 mins only) is a relatively harsh treatment, but also stated that very similar results were achieved from overnight incubation in nuclear isolation buffer at 4°C, a standard protocol in biochemical analysis (where material may be stored for weeks in this manner). We would also agree that without confirmatory thin sections we cannot be sure whether or not the entire outer nuclear membrane and pore complex have been retained. In comparison with the work of Kirschner et al (1977) it appears that we have not retained the full nuclear pore complex (Figs. 21-22). However, as an indication that we have exposed the cytoplasmic/nuclear interface, the appearance of remnants of the nuclear pore system is considered a useful signpost. We would also agree that there are no obvious central granules remaining.

Reviewer II: In Fig. 15, are the authors convinced that all of these various-sized fibres are chromatin or is there a possibility of non-nuclear contamination? Nuclear lamina contamination? Or, the presence of nuclear matrix remnants?

Authors: We feel that the successive removal of nuclear peripheral material (Figs. 8 through 12) probably leaves only the nuclear contents remaining (see also Figs. 21-22). Recent preliminary experiments using detergents specific for solubilisation of non histone chromosomal proteins and/or membrane proteins, [i.e., Zwitterionic detergents SB 12, SB 14 and Chaps; (Matno Y, Matsui S, Nishi N, Wada F, Sandberg AA: Analytical Biochemistry 150, 337-344, 1985)] have shown very similar results to Joy, Sarkosyl and SDS.

D.H. Welter: In Fig. 3 the fibres forming the fibrous network appear to be larger than the 11 nm fibre reported by others to make up the nuclear shell. What is your morphological basis for determining that these are intermediate filaments forming this network and what relationship do they have to the underlying 30 nm chromatin fibre?

Authors: Our suggestion for the fibrous material to be composed of intermediate filaments was based on the idea of these elements of the cytoskeleton being generally

accepted as the most likely to be nuclear associated from the intermediate filament/microtubule/microfilament complex which forms the whole cytoskeleton (see Figs. 21-22). Without immunocyotchemical characterisation, obviously we cannot be certain. With respect to the increased diameter, this could be due to either aggregation or cytoplasmic contamination of the fibres themselves. We have not yet elucidated whether or not there is any direct relationship between this network and the chromatin in the nucleus, although we would expect that such a relationship might well be interfaced by the nuclear lamina.

D.H. Welter: In Fig. 15 there are three distinct fibre diameters and you reference them as all being chromatin. If these are all photographed near the nuclear surface could they not contain intermediate filaments, particularly those in the 10 nm range?
Authors: We are confident that the detergent extraction to which the material in Fig. 15 has been subjected was sufficient to remove everything but nuclear contents. Consequently (see response to Q3, Adolph) we feel that these fibres do in fact represent different orders of packaging of chromatin.

G.H. Haggis: The main thrust of the results presented in this paper is that, as we go through Figs. 1, 2, 7, 9, 10 and 12, successive layers of structure are being removed to give views of increasing depth into the nuclear structure. However, we need more evidence from thin-section and/or biochemical study, as to whether, for example, Triton treatment in the preparation of Fig. 1 may not have already removed both membranes of the nuclear envelope (including ribosomes on the outer membrane). We may be already looking at the lamina, or at peripheral chromatin. On the other hand, in Fig. 9, we might be looking at dispersed lamins rather than peripheral chromatin. Would the authors agree that, while they have developed good methods for gradual disruption of the nucleus, further work is needed before we can be certain which element of nuclear structure we are viewing in many of these micrographs.
Authors: We agree absolutely with this comment. However, by showing both end points, we do feel that the right protocol, (supported by both thin section and immunocytochemical confirmation of the presence or removal of nuclear lamins), should result in the surface viewing of the structure of this interface in a manner much more likely to help elucidate its function. This 'biochemical dissection' approach may prove to be considerably more involved than merely judging the right time of detergent extraction, and quite easily could involve nuclear isolation, brief extraction, stabilisation using protein crosslinkers such as EGS. (Blow J and Watson J V, Embo J. 6: 1997-2002, 1987) and further subsequent extraction, according to which structures were desired to be retained. We also plan to run parallel and probably identical gels from each coverslip preparation to analyse the specificity and amounts of extracted components. This first paper hopefully points the way towards unravelling some of the functional structure within the nucleus which to date, has largely eluded those investigators committed to the 'two dimensional' approach of thin sectioned and/or spread material.