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PROBLEMS IN PREPARATION OF CHROMOSOMES FOR SCANNING ELECTRON MICROSCOPY TO REVEAL MORPHOLOGY AND TO PERMIT IMMUNOCYTOCHEMISTRY OF SENSITIVE ANTIGENS

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Abstract

Although much information about chromosome structure and behaviour has been obtained using light microscopy, greater resolution is needed for a thorough understanding of chromosome organisation. Scanning electron microscopy (SEM) can provide valuable data about these three-dimensional organelles. The introduction of methods using osmium impregnation of methanol-acetic acid-fixed chromosome spreads revolutionised matters, producing life-like images of chromosomes. Nevertheless, it became clear that osmium impregnation introduced various artefacts, although the resulting images were still useful. Methanol-acetic acid-fixed chromosomes are, in fact, flattened on the glass substratum, and the 3-dimensional appearance obtained after osmium impregnation is the result of swelling during this process. At the same time, the fibrous substructure of the chromosomes becomes much coarser. More recently a number of alternative methods have become available for studying chromosomes by SEM. Isolated chromosomes, that have not been allowed to dry during preparation, retain a 3-dimensional appearance without osmium impregnation, and the same is true of methanol-acetic acid-fixed chromosomes that have been treated with 45% acetic acid and processed without drying; however, these methods do not permit the routine production of intact metaphase spreads. Use of cytocentrifuge preparations obviates the use of acetic acid fixation and osmium impregnation, produces intact metaphase spreads, and permits the immunocytochemical detection of antigens that are easily destroyed by routine fixation procedures.

Key Words: Chromosomes, scanning electron microscopy, immunocytochemistry, methanol-acetic acid fixation, osmium impregnation.

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Introduction

Because of their 3-dimensional structure, disposition in the cell, and behaviour, chromosomes should be highly appropriate objects for study by scanning electron microscopy (SEM). However, it has not proved to be a simple matter to prepare chromosomes for SEM, as this necessarily involves freeing them from the surrounding cytoplasm, with the probability of altering their structure. In fact, early attempts to examine chromosomes by SEM provided little useful information (Christenhuss *et al.*, 1967; Neurath *et al.*, 1967; Smith 1970; Pawlowitzki and Blaschke, 1971). The introduction of osmium impregnation techniques, which provide apparently lifelike images of chromosomes, represented a great advance (Harrison *et al.*, 1981; Maruyama 1983; Mullinger and Johnson 1983; Takayama *et al.*, 1985), and a substantial amount of work has been done using these procedures. There are, nevertheless, grounds for supposing that the osmium impregnation methods may introduce a number of artefacts. For a start, the chromosomes have to be fixed in methanol-acetic acid, which is known to extract histones and other proteins from chromosomes (Dick and Johns, 1968; Sivak and Wolman, 1974; Retief and Rüchel, 1977; Hancock and Sumner, 1982), and in general renders them unsuitable for immunocytochemical procedures. Secondly, the results of impregnating chromosomes with osmium can be quite variable, and there is evidence that a significant part of this variability could be due to the osmium impregnation itself (Sumner and Ross, 1989; Sanchez-Sweatman *et al.*, 1993). It is therefore desirable to consider carefully what artefacts might be produced during preparation of chromosomes for SEM.

In the work to be described in this paper, changes that occur in chromosome morphology during osmium impregnation of methanol-acetic acid-fixed chromosomes for SEM are described, and alternative methods of chromosome preparation, that may preserve morphology or immunogenicity better, are investigated. It has been a particular concern to preserve the immunogenicity of certain antigens (the kinetochore antigens that react with

CREST serum - Tan, 1989, and the antigen recognised by AC1 - Holland *et al.*, 1995) that are easily destroyed by fixation. Preliminary results show that with suitable methods of preparation, immunolabelling of sensitive antigens on chromosomes prepared for SEM can be carried out, thus adding compositional information to purely morphological observations.

Material and Methods

Chromosome preparations

Conventional methanol-acetic acid fixed chromosome spreads were made from human lymphocyte cultures according to standard procedures (e.g., Watt and Stephen, 1986; Macgregor and Varley, 1988), or from CHO (Chinese hamster ovary) cells, cultured in RPMI 1640 medium until nearly confluent, and accumulated in metaphase using Colcemid. After making the spreads on 22 mm square coverslips, they were allowed to dry, usually overnight, before processing further.

For treatment with 45% acetic acid, the above procedure was modified using a method derived from that described by Martin *et al.* (1994). Chromosome preparations from CHO cells, fixed in methanol-acetic acid, were spread on coverslips in the usual way, but instead of letting the cell suspension dry, the coverslips were flooded with 45% acetic acid immediately before the methanol-acetic acid finally dried out. After a few seconds they were plunged into glutaraldehyde (2.5% in cacodylate buffer, pH 7.4, containing 0.1M sucrose), left overnight, and either dehydrated and critical point dried from carbon dioxide, or impregnated with osmium as described below.

Cytocentrifuge preparations were also made from cultures of human lymphocytes or CHO cells, grown in the same way as for methanol-acetic acid fixation. However, treatment at the end of culture was different. After pelleting the cells and decanting off the supernatant, the cells were resuspended in the hypotonic solution described by Stenman *et al.* (1975) for 10 min in the refrigerator. This hypotonic solution consists of 10 mM HEPES[4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, sodium salt], 30 mM glycerol, 1 mM calcium chloride, and 0.8 mM magnesium chloride. After this treatment, 0.3 ml of the cell suspension was added to each chamber of a Shandon Cytospin centrifuge, and the cells were spun down on to slides for 15 min at 1500 rpm. After centrifuging, the slides were allowed to dry, and either used immediately, or left overnight before further processing.

Isolated human chromosomes were prepared using the polyamine method of Sillar and Young (1981). The resulting chromosome suspension was diluted approximately 5-fold with PBS (phosphate-buffered saline, Oxoid). Coverslips of 13 mm diameter were loaded into the wells of a multiwell plate (Falcon 24-well plate, cat. no. 3047, Becton Dickinson), and approximately 0.5 ml of the chromosome suspension added. The multiwell plates were then centrifuged for 10 min at 1000 rpm in a Sorvall ST6000 refrigerated centrifuge at 0-4°C. Subsequent processing was carried out without letting the specimens dry, by pipetting off the supernatant and adding the next solution to the well.

Further treatments

Methanol-acetic acid fixed chromosome spreads were treated with trypsin (Difco Bacto trypsin, reconstituted according to the manufacturer's instructions, and then diluted further 100-fold with distilled water). This solution was always used fresh, and digestion of chromosome preparations was for 5-30 s. After digestion, the chromosome preparations were washed thoroughly with distilled water. Some preparations were left undigested with trypsin. The slides were then transferred to glutaraldehyde for further processing (see below).

Cytospin preparations were treated with Triton X-100 (0.1% in PBS) for 5, 15 or 30 min, to remove cytoplasm, and then washed in PBS (3 lots, each for 5 min), before fixation with glutaraldehyde or immunocytochemical labelling (see below).

Isolated chromosomes were transferred to glutaraldehyde before osmium impregnation (see below) or critical point drying.

Osmium impregnation

Chromosome preparations were left in glutaraldehyde (2.5% in cacodylate buffer, pH 7.4, containing 0.1 M sucrose), either for 30 min or overnight, whichever was more convenient for that particular experiment. After washing thoroughly in tap water, the preparations were transferred to freshly prepared osmium tetroxide (1% in distilled water, 5 min), and then again washed very thoroughly with running tap water, before transfer to a freshly prepared solution of thiocarbohydrazide (TCH, 0.5% in distilled water, 5 min), followed by another thorough wash in running tap water. Thiocarbohydrazide acts as a bifunctional ligand, binding to osmium already in the tissue, and in turn binding further osmium at the next stage of osmication (Murphy, 1978). This cycle of osmium tetroxide and TCH was repeated several times (3 to 11 times, according to the requirements of the experiment), always finishing with an osmium tetroxide treatment. In experiments in which the number of cycles of treatment was not varied, it was standardised at nine.

After the last wash, the specimens were dehydrated through graded acetone solutions (25%, 50%, 75% and 100%), and critical point dried from liquid carbon

dioxide. The slides or coverslips were broken into small enough pieces, and the pieces bearing chromosomes attached to stubs with double-sided adhesive tape. They were then coated lightly with platinum in a Polaron E5100 sputter coater, and examined in a Hitachi S-800 field emission scanning electron microscope, at accelerating voltages between 1 and 25 kV.

Immunocytochemistry

CREST serum was obtained from Professor G. Nuki (Department of Rheumatology, University of Edinburgh) and before use was diluted at between 1:25 and 1:50 with PBS containing 1% bovine serum albumin (BSA). Monoclonal antibody AC1 (Holland *et al.*, 1995) was a gift from Dr. G. Hadlaczky (Institute of Genetics, Biological Research Centre, Szeged, Hungary), and was used without dilution. Chromosome preparations were incubated with the antibody solution overnight (up to 19 h), and then washed with PBS containing 1% BSA (3 × 5 minutes). Preparations treated with CREST serum were then incubated for approximately 2 h with horseradish peroxidase (HRP)-labelled anti-human IgG (Sigma) diluted at between 1:25 and 1:50 in PBS containing 1% BSA, while chromosomes that had been incubated with monoclonal antibody AC1 were incubated with HRP-labelled anti-mouse IgM (Sigma) diluted as above. After this the slides were washed again in PBS containing 1% BSA (3 × 5 minutes). Peroxidase activity was detected using diaminobenzidine (DAB) solution (Sigma: 0.5 mg/ml in PBS), to which 50 μl of 1 volume hydrogen peroxide was added immediately before use. Incubation was for 1 h, after which the reaction product was intensified with silver, as described by Burns *et al.* (1985).

For colloidal gold labelling, chromosome preparations which had been incubated with monoclonal antibody AC1 were transferred to Tris-HCl buffer, pH 8.2, containing 0.9% sodium chloride and 1% BSA, and then incubated with anti-mouse IgM labelled with 10 nm colloidal gold (British Biocell International, Cardiff, UK) diluted 1:25, for 2 h. After incubation the chromosomes were washed again in the Tris buffer, then in distilled water, and the colloidal gold particles enhanced using a Silver Enhancement Kit (British Biocell International), according to the manufacturers instructions.

Immunolabelled chromosome preparations were dehydrated and critical point dried as described above, without osmication.

Results

Morphology of methanol-acetic acid fixed chromosomes

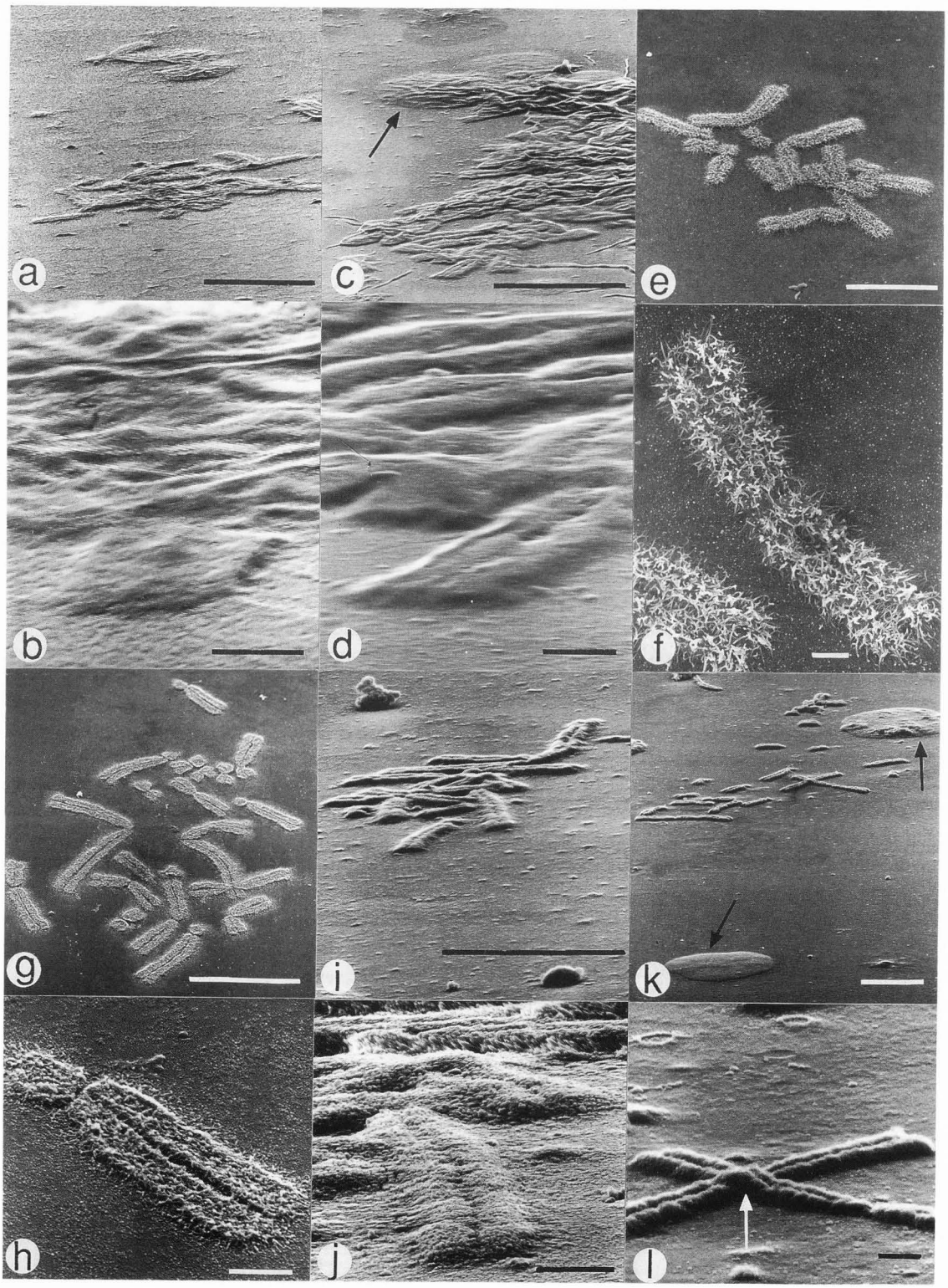
Chromosomes fixed in methanol-acetic acid, spread on glass, and prepared for SEM without further treatment are only slightly raised and show no fine structure (Fig. 1 a, b), in agreement with early scanning electron microscope observations of chromosomes, in which there was no osmication (Christenhuss *et al.*, 1967; Neurath *et al.*, 1967; Smith, 1970; Pawlowitzki and Blaschke, 1971). The chromosomes appear similar if they are fixed in glutaraldehyde before critical point drying (Fig. 1 c, d); note that interphase nuclei are only very slightly raised and do not appear as the expected nearly spherical objects. If, however, the chromosomes are treated briefly with trypsin, or indeed merely washed with PBS, before glutaraldehyde fixation and critical point drying, the chromosomes are still relatively flattened, but are seen to consist of a network of fine fibres (Fig. 1 e, f), as described by Squarzoni *et al.* (1994) and by Rizzoli *et al.* (1994). Subsequent impregnation with osmium results in the disappearance of the fine fibrillar structure, to be replaced with a more granular appearance; at the same time the profile of the chromosomes becomes raised, with an approximately semi-circular cross-section (fig. 1 g-l). Note that with the maximum degree of osmium impregnation, the nuclei remain relatively flattened (Fig. 1k), while chromosomes cross each other in a way that appears most unnatural (Fig. 1l).

Size of fibres in methanol-acetic acid fixed chromosomes

Sizes of chromosome fibres were measured on high resolution micrographs taken at ×120 000. No significant differences were found between the sizes of fibres imaged at 5 kV or 25 kV, or between coated and uncoated fibres. Chromosomes subjected to increasing numbers of stages of osmication showed a steady increase in the size of their fibres, or, for the more heavily osmicated chromosomes, their surface granularity (Fig. 2). On the other hand, trypsin treatment before osmium impregnation produces a progressive decrease in the size of the chromosome substructures (Fig. 3). Note that in all cases there is substantial variability in the size of the objects being measured, as well as differences between separate experiments. Nevertheless, the general trends are repeatable.

Isolated chromosomes

Chromosomes isolated using the polyamine method appear to be well raised above the glass substratum, with



a circular cross-section, but tend not to show a clear split into sister chromatids (Fig. 4). Chromosomes prepared by critical point drying without osmium impregnation (Fig. 4a) appear generally similar to those that have received osmication (Fig. 4 b, c), although the unosmicated chromosomes appear slightly smoother.

Figure 1 on facing page

Figure 1. Human lymphocytes chromosomes, fixed in methanol-acetic acid, and prepared for scanning electron microscopy. **a,b**: chromosomes spread on glass and examined without further treatment; the chromosomes are flattened and featureless. **c,d**: chromosomes fixed in glutaraldehyde and critical point dried; the appearance is quite similar to that of untreated chromosomes. Note the flattened appearance of the interphase nuclei (arrow). **e,f**: chromosomes digested with trypsin, 5 sec, fixed in glutaraldehyde, and critical point dried; these chromosomes show a network of fine fibres. **g,h**: chromosomes digested with trypsin, fixed in glutaraldehyde, and impregnated with 3 cycles of osmium tetroxide/-thiocarbohydrazide (OTOTO) treatment. Chromosomes similar to those in e and f, without osmium impregnation. **i,j**: as g and h, but with 5 cycles of OTOTO; the chromosomes are distinctly raised with a semi-circular profile. **k,l**: as g-j, but with 7 cycles of OTOTO; the chromosomes are raised still higher than in i and j, but nuclei are still flattened (arrows in k), and chromosomes cross each other in an unnatural-looking way (arrow in l). Scale bars equal 10 μm in a, c, e, g, i, and k and equal 1 μm in b, d, f, h, j and l.

Chromosomes prepared with 45% acetic acid

Methanol-acetic acid fixed chromosomes treated with 45% acetic acid immediately before drying, and then plunged into glutaraldehyde, show good morphology, with an approximately circular cross-section (Fig. 5). Chromosomes overlap each other in a natural way (Fig. 5a), in contrast to the appearance given by the standard methanol-acetic acid/osmication procedure (Fig. 1l). Unosmicated chromosomes appear relatively smooth (Fig. 5a), while osmicated chromosomes have a more fluffy appearance. Complete metaphases are rarely seen. Interphase nuclei are well raised, with an approximately spherical shape (Fig. 5c), in complete contrast to nuclei prepared by the standard method (e.g. Fig. 1k).

Cytocentrifuged chromosomes

Metaphase cells prepared by cytocentrifugation do not reveal chromosomes unless the surrounding cytoplasm is removed. Treatment with Triton X-100, followed by glutaraldehyde fixation and critical point drying, shows chromosomes that are only slightly raised above the surrounding material, and that are largely featureless (Fig. 6a). Subsequent osmication, however, produces metaphases in which the chromosomes have a good 3-dimensional structure and show a fibrous substructure (Fig. 6 b, c).

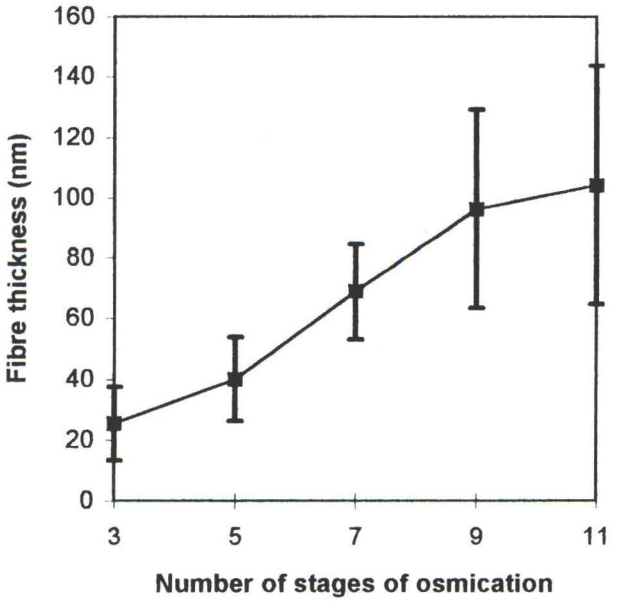


Figure 2. Graph showing the increase in thickness of chromosome fibres with increasing number of cycles of osmication. Methanol-acetic acid-fixed chromosomes, no trypsin treatment. Error bars represent ± 1 standard deviation.

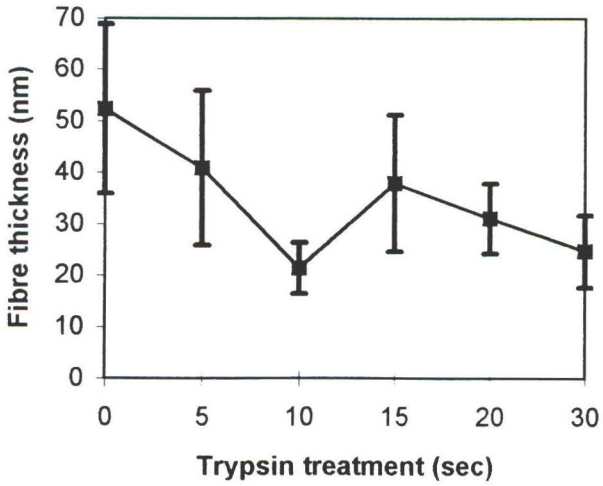


Figure 3. Graph showing the decrease in thickness of chromosome fibres with increasing length of trypsin treatment. Methanol-acetic acid-fixed chromosomes, subjected to 9 cycles of osmication after the trypsin treatment and glutaraldehyde fixation. Error bars represent ± 1 standard deviation.

Immunocytochemistry

CREST serum labels the kinetochores of mammalian chromosomes (see Tan, 1989, for a review). When applied to unfixed cytocentrifuge preparations of human

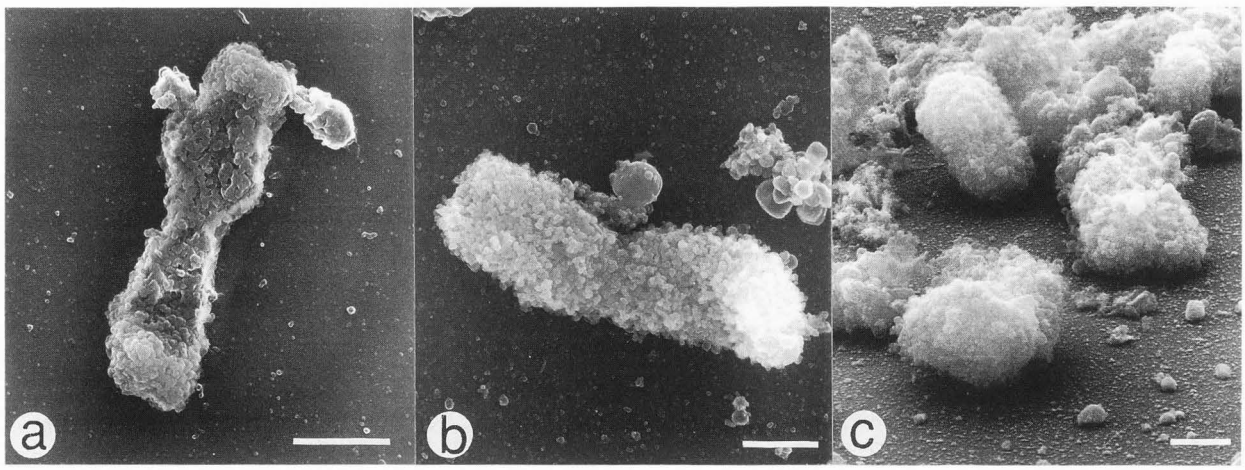


Figure 4. Scanning electron micrographs of isolated chromosomes. **a**: critical point dried without any prior treatment. **b**: osmicated before critical point drying. **c**: as b, showing that the chromosomes are approximately circular in cross-section. Scale bars = 1 μm.

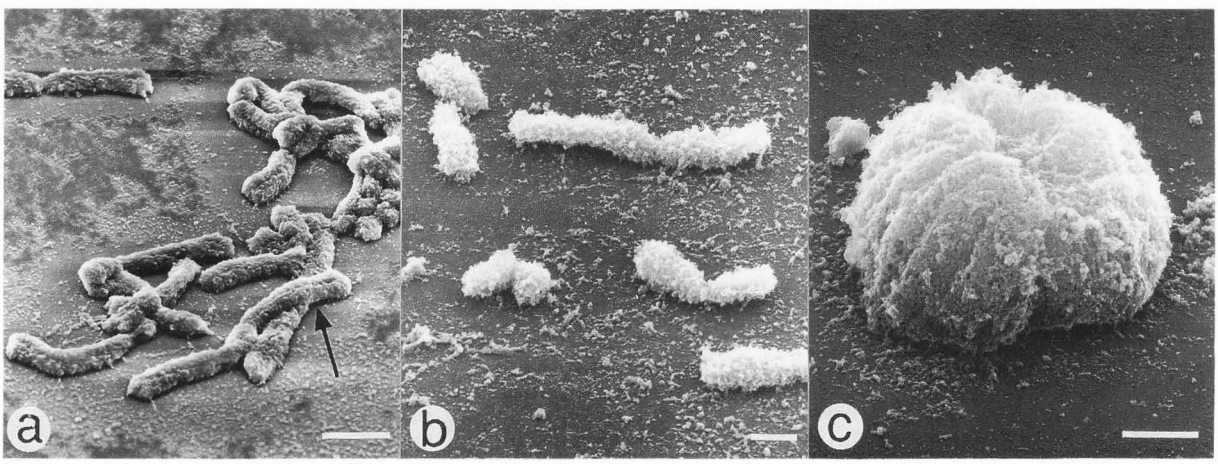


Figure 5. Methanol-acetic acid-fixed chromosomes, spread on glass, and treated with 45% acetic acid, and not allowed to dry. **a**: non-osmicated chromosomes; note the approximately circular cross-section, and the natural way in which one chromosome lies across another (arrow). **b**: osmicated chromosomes; similar to a, but with a fluffier appearance. **c**; a nucleus from an osmicated preparation; note that it is well raised and approximately spherical, not flattened like those shown in Figure 1. Scale bars = 2 μm.

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chromosomes, and detected using a horseradish peroxidase-labelled secondary antibody, silver intensification of the DAB reaction product revealed paired dots at the centromeres (Fig. 7a), just as can be demonstrated by, for example, immunofluorescence. These dots are clearly visible using back-scattered electrons (Fig. 7 a, c), but are virtually invisible using the secondary electron signal (Fig. 7b). Chromosome structure is reasonable even though the chromosomes were not fixed before labelling, and no osmication was carried out afterwards.

Monoclonal antibody AC1 labels another centromeric antigen, which in some cases appears, by fluorescence microscopy, to form a ring round the centromeric constriction (Holland *et al.*, 1995), although the resolution is scarcely good enough to be confident of this. Such a centromeric ring can be seen clearly on the chromosome shown in Fig. 7d, and is especially clear when a series of micrographs at different angles of tilt is examined. In Fig. 7e, labelling is demonstrated using back-scattered electrons: on some chromosomes it extends right across the centromeres, while on others it forms two discrete blocks, one on each side. In both Fig. 7d and 7e, the area occupied by the DAB reaction

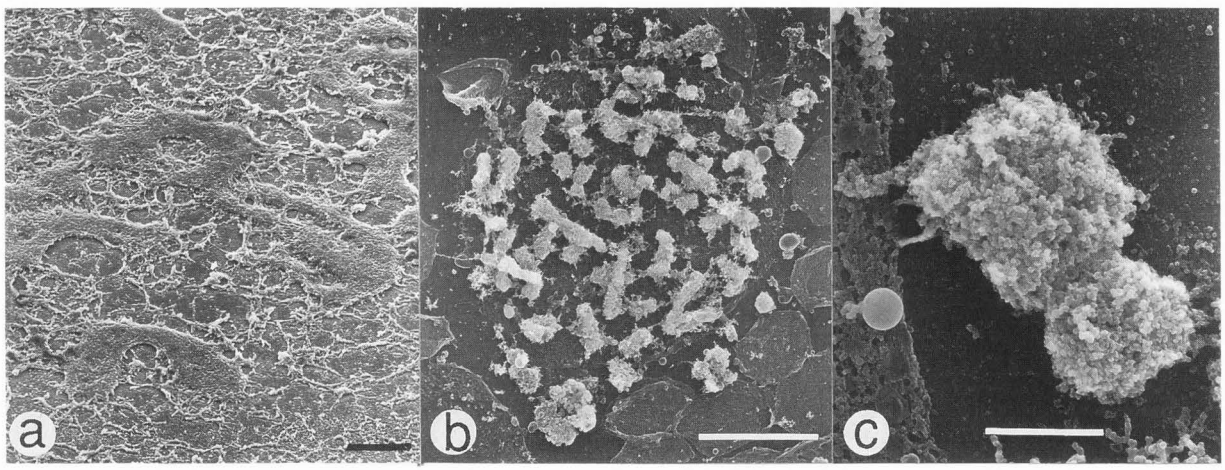

Figure 6. Cytocentrifuged preparations of chromosomes. **a**; CHO chromosomes treated with Triton X-100, fixed in glutaraldehyde and critical point dried. The chromosomes are only slightly raised above the substratum. Scale bar = 2 μm. **b**: human chromosomes treated with Triton X-100, fixed in glutaraldehyde, and subjected to 5 cycles of osmication. The chromosomes are now well raised. Scale bar = 10 μm. **c**: a single chromosome treated as in b, showing the fibrous substructure. Scale bar = 1 μm.

product is quite extensive, and in Fig. 7e also rather diffuse, as if the DAB reaction product has spread from the actual site of labelling. Results with an alternative method of labelling, silver-enhanced colloidal gold, are shown in Fig. 7f. Although there is considerable, presumably non-specific, scatter of colloidal gold particles, at least two regions are visible where there is a concentration of gold particles extending across the width of the chromosomes.

Discussion

Chromosomes fixed in methanol-acetic acid, and spread on glass, appear featureless and only very slightly raised above the substratum, when examined by SEM. Similar results have previously been reported by others who have examined fixed, but otherwise untreated chromosomes by SEM (Christenhuss *et al.*, 1967; Neurath *et al.*, 1967; Smith, 1970; Pawlowitzki and Blaschke, 1971) and observations using Atomic Force Microscopy show that such chromosomes are between 50 nm and 350 nm high (de Groot and Putman, 1992; Fritzsche *et al.* 1994). Exposure of the fixed chromosomes to buffer (with or without trypsin) reveals a network of fibres throughout the chromosomes (as described by Squarzoni *et al.* (1994) and by Rizzoli *et al.* (1994), but this tends to be obscured by subsequent osmium impregnation, which also swells the chromosomes. In fact, exposure of chromosomes to phosphate-buffered saline (PBS) also results in their height increasing, to between 300 nm and 900 nm (de Groot and Putman, 1992; Fritzsche *et al.*, 1994). Osmium impregnation has also been shown to deposit enough material on subcellular structures to produce a significant increase in size (Kelley *et al.*, 1973; Ip and Fischman 1979), which could well account for the increase in size of chromosome substructure reported here, as well as the qualitative change in surface structure observed. Although the continuous, granular appearance of heavily osmicated chromosomes might simply be the result of gross swelling of individual fibres, which as a result have become fused to form a continuous but rough surface, other explanations are possible. Rizzoli *et al.* (1994) regard the surface as being formed from precipitates of osmium with the thiocarbohydrazide used for impregnation. On the other hand, it is now well established that chromosomes do have a surface coating of ribonucleoprotein (Hernandez-Verdun and Gautier, 1994), and it has been proposed that this forms the surface coat seen after osmium impregnation (Sumner and Ross, 1989). Thus there remain many problems in interpreting the images obtained by SEM from methanol-acetic acid-fixed chromosomes. Although they appear much as one might expect, they are clearly the result of a series of artefactual changes, and cannot be a precise representation of the morphology and fine structure of the chromosomes as they were in life. Although much valuable work has been done with such preparations, results must be interpreted with caution, and probably it is only at the grossest level of chromosome structure that reliable conclusions can be drawn. In particular, the dimensions of the fibrous or granular substructure seen after osmium impregnation are very variable, and are dependent on both the degree of osmium impregnation

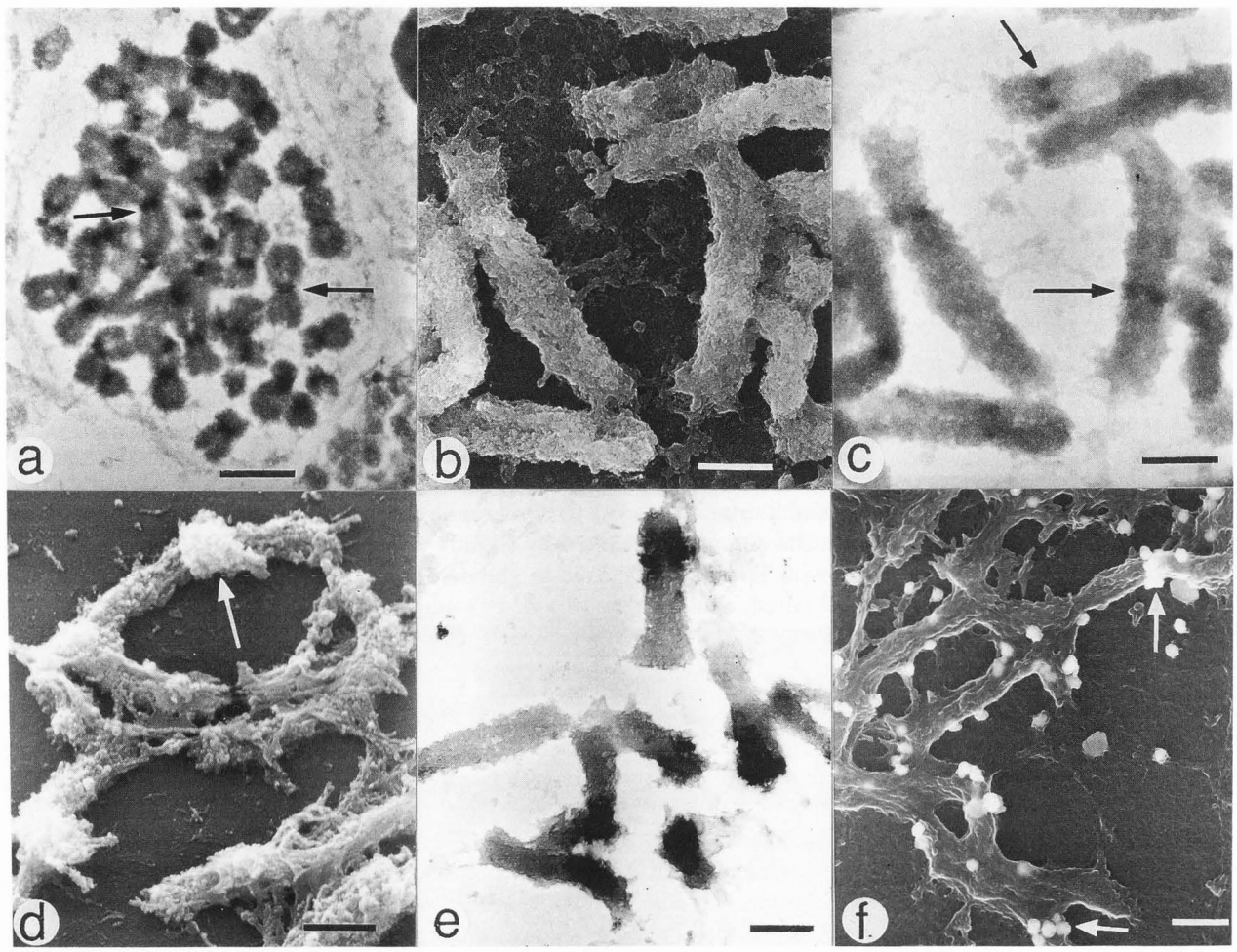

Figure 7. Scanning electron micrographs of immunolabelled human chromosomes, prepared by cytocentrifugation. **a-c**: CREST labelling, detected using horseradish peroxidase (HRP)-labelled second antibody, diaminobenzidine (DAB), and silver intensification. a: metaphase spread, imaged using back-scattered electrons (BSE) in reverse contrast (i.e. strong signals appear dark). Note the reaction at the centromeres of the chromosomes (arrows). Scale bar = 5 μm. b and c: the same chromosomes imaged using secondary electrons (b) and BSE (c); the reaction product is clearly visible as two spots at the centromeres using BSE (arrows), but is scarcely visible using secondary electrons. Scale bars = 2 μm. **d-f**: chromosomes labelled with monoclonal antibody AC1. d and e: antibody detected using HRP, DAB and silver intensification. In d, the reaction product is visible using secondary electrons as a substantial ring round the centromere (arrow), while in e, using BSE, the reaction appears as a diffuse mass. Scale bars = 1 μm. f: sites of antibody binding detected using colloidal gold, followed by silver intensification, and imaged using BSE. Sites of apparently specific labelling arrowed. Scale bar = 1 μm.

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and the amount of trypsin treatment.

The flattening of chromosomes on to the substratum can be avoided if the chromosome spreads are never allowed to dry until preparation is finished (i.e. when critical point drying is completed). This can be achieved either by using isolated chromosomes, in which case it is impossible to obtain metaphase spreads, or by using treatment with 45% acetic acid. The latter procedure, pioneered by Martin *et al.* (1994), not only involves the use of quite concentrated acetic acid, but also causes disruption of many metaphases, although the chromosomes do appear to have a lifelike configuration. With both the isolated chromosomes and those prepared using 45% acetic acid, osmium impregnation seems to produce little change to the general morphology of the chromosomes.

Of the methods discussed so far, only the use of isolated chromosomes is likely to be compatible with immunocytochemical labelling, since methods involving acetic acid will destroy or extract most chromosomal

antigens (Jeppesen, 1994). However, the use of isolated chromosomes for immunocytochemistry in the SEM has not been pursued, since the impossibility of obtaining metaphase spreads makes identification of specific chromosomes more difficult. Cytocentrifuge preparations are usually used for light microscopical immunocytochemistry of chromosomes (Jeppesen, 1994), and it has proved practicable to use similar preparations for SEM. The main difference between making cytocentrifuge preparations of chromosomes for light microscopy and for SEM is that in the latter case it is necessary to remove the surrounding cytoplasm to visualise the chromosomes; use of a mild detergent such as Triton X-100 is adequate to achieve this, and does not appear to cause any morphological degradation of the chromosomes, nor does it extract the antigens that have been tested. On the other hand, it is still not entirely clear how well preserved the chromosome morphology is. There is some evidence that the centrifugation process may leave the chromosomes somewhat flattened, even though they are surrounded by a supporting layer of cytoplasm. Nevertheless, well raised chromosomes with good morphology can be obtained without osmication. Although such preparations clearly make possible the immunolabelling of specimens to be viewed in the SEM, with the advantage of being able to study the 3-dimensional arrangement of antigens on chromosomes, further refinement of the chromosome preparation, resulting in greater consistency, and optimisation of the immunolabelling process, are clearly desirable. While horseradish peroxidase in combination with DAB can produce a strong, clear reaction, with apparently minimal non-specific background, it appears to be essentially a low resolution technique, owing to spreading of the DAB reaction product during development. On the other hand, colloidal gold, although it should theoretically produce much higher resolution, limited only by the size of the gold particles, seems to be prone to producing excessive background labelling on this type of specimen, and further work is required to optimise labelling.

From the point of view of producing chromosome preparations for SEM with the minimum of treatment that might disrupt structure or extract chromosomal components, cytocentrifuge preparations are probably best, but the metaphases so produced tend to lack the clarity of conventional metaphases fixed in methanol-acetic acid and impregnated with osmium. It is, however, clear that the latter type of preparations involve so many artefacts that they cannot be acceptable for studying anything more than the grosser features of chromosome organisation. In fact, osmium impregnation does not seem to be necessary for obtaining good chromosome preparations for the SEM or even for producing adequate contrast. Preparations of chromosomes made by treatment with 45% acetic acid, without drying, seem to be superior morphologically to standard air-dried, methanol-acetic acid-fixed spreads, but so far it has proved to be difficult to retain intact metaphases from mammalian cells, presumably because drying is important for the adherence of the chromosomes to the substratum. Perhaps a universal method of preparing chromosomes for SEM that retains them in the configuration that they had in life, while permitting immunolabelling of even the most delicate of antigens, is not possible, and it may, for the foreseeable future, be necessary to use specific procedures depending on the application required.

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Discussion with Reviewers

B. Hamkalo: Why is the chromosome overlap shown in Fig. 1l considered "unnatural"?
Author: The chromosome indicated appears to be floppy, that is, it has no natural stiffness. Configurations seen in living and in fixed cells suggest that bends of the kind illustrated do not occur naturally, and the chromosome in Fig. 5a also appears to be stiffer. The implication is that the chromosome in Fig. 1l has either been distorted during the drying process after spreading, or has been rebuilt in an artificial configuration during osmication.

B. Hamkalo: Does the author have an explanation for why PBS treatment results in so much better definition in the methanol-acetic acid-fixed preparations?
Author: It is assumed that washing the fixed chromosomes in PBS (or indeed probably almost any buffer) washes away a layer of material, perhaps cytoplasmic protein, but possibly a superficial layer of the chromosome itself (see Hernandez-Verdun and Gautier, 1994), thereby exposing the chromosomal fibres. So far as I know, this hypothesis has not been formally tested.

E. De Harven: With reference to Figure 2, do you imply that the average diameter of chromosomal fibres

after three stages of osmication is only 30 nm? If this is the case, what do you regard as the diameter of the un-osmicated fibre?

Author: In the particular experiment the results of which are illustrated in Figure 2, un-osmicated specimens were not prepared. In specimens that have not been treated in any way after spreading on glass, no fibres are visible. Treatment with trypsin or PBS, followed by glutaraldehyde fixation, give fibre diameters in the region of 20-30 nm. This would suggest that (i) methanol-acetic acid-fixed chromosomes show much the same fibre diameter that would be expected in a native chromosome, and (ii) that **light** osmication does not cause any significant increase in fibre diameter. There is, however, some variation between and within individual experiments, and it would not be justifiable to combine results from separate experiments, although trends found in repeated experiments of the same type are reproducible.

B. Hamkalo: The morphology of chromosomes in Fig. 7f is considerably poorer than others shown in this figure. Is this due to the additional steps required for immunogold labelling and intensification? If so, has the author tried to introduce a brief fixation step prior to adding immunogold or to modify chemically the primary antibody (with, for example, biotin) so that after this reaction chromosomes could be fixed with glutaraldehyde and detected with streptavidin gold?

Author: The relative degradation of morphology seen in Fig. 7f could well be due the additional treatments that this specimen was subjected to, although there is quite a bit of variation in this type of preparation regardless of the immunolabelling treatment used. It would certainly be valuable to try the modifications suggested, and it might well be expected that an intermediate fixation stage would result in improved morphology at the end of the procedure. Obviously, in any procedure as complicated as this, the number of possible variations is rather large, and it has not been possible to try all the useful ones yet.

B. Hamkalo: Given all the morphological changes described, what does the author think about the chances of defining the *bona fide* structure of the chromosome?

Author: This is a very speculative matter! It is relatively easy to suggest what the best procedures to maintain a lifelike image of chromosomes would involve. Firstly, it is clearly necessary to avoid flattening on to the substrate, and this almost certainly means that drying must be avoided. Secondly, the surrounding cytoplasm must be removed, without extracting chromosomal components or altering chromosome structure. Thirdly, it will be necessary to find some reliable way of attaching to the substrate chromosomes prepared according to these principles. If a number of independent procedures, based on such principles, produce similar images of chromosomes, then it is probably reasonably safe to say that a good representation of chromosome organisation has been attained.

T.D. Allen: You rightly state that 3:1 methanol-acetic fixation and spreading results in chromosome flattening, and that cytocentrifugation obviates this step. However, in your materials and methods, you state that after centrifuging, the slides were left to dry - surely air-drying in this situation is going to flatten the chromosomes via the surface tension as the air-water interface passes through the chromosomes to a far greater effect than the evaporation of the more volatile methanol-acetic mixture (75% methanol). While avoiding the problems of methanol-acetic fixation, you have replaced them with air-drying from an aqueous medium. In our own experiments, we have deliberately 'overfilled' the cytocentrifuge well, and carried out the preparation direct from moist without air-drying. This would also perhaps remove the need for detergent extraction that you state for your cytocentrifuge preparations.

Author: Your point is well taken - drying of any sort would be best avoided, although it clearly helps to attach cells and chromosomes to the substrate. With cytocentrifuge preparations, it was suspected that cytoplasmic proteins might nevertheless help to support the chromosomes, although the image in Fig. 6a casts doubt on this. I suspect that, however the chromosomes might be prepared by cytocentrifuging, detergent extraction of cytoplasm would still be necessary. On an historical note, the method described for preparing chromosomes for scanning electron microscope immunocytochemistry was derived directly from methods used for light microscope immunocytochemistry; in view of the sensitive nature of the antigens being investigated, it was necessary to proceed cautiously, and adapt existing procedures for light microscopy to the scanning electron microscope, without attempting any radical new approaches.

T.D. Allen: Does it really need nine repetitions to be consistent in osmium impregnation? During the later stages of our own studies, we reduced the protocol to OsO_4 fix, TCH impregnation, OsO_4 fix, i.e. one cycle only.

Author: In practice we determine the number of cycles of osmium impregnation empirically, and the optimal degree of impregnation varies across the specimen, and from one specimen to another. Precise counting of the number of cycles of osmication is obviously necessary for the sort of studies described in this paper, but for routine use, the osmication is repeated until the techni-

cian thinks it 'looks right'. I suspect the number of cycles of osmication required may also be influenced strongly by the precise details of the methanol-acetic acid-fixation, which is something that appears to vary considerably from one laboratory to another.

T.D. Allen: 'Lightly coated' with platinum. How thick was this coating - platinum is known to be grainy and not to form a continuous coat with maybe 3-4 nm thickness at least.
Author: Partly because of the problems mentioned by the questioner, we did not attempt to measure the thickness of the platinum coating. We suspect that it would in fact be very variable on the rough and fibrous surfaces that we were dealing with. In practice, we found no differences in detailed appearance or in dimensions of the surface structure of the chromosomes whether they were coated or not, suggesting that the coating was very thin. On a technical point, it appeared that the coating was only necessary to provide conductivity across the glass substrate; uncoated chromosomes that had been spread on pre-coated glass coverslips did not charge up.

T.D. Allen: DAB as the end point is not a 'point' localisation, as colloidal gold is - why was it chosen in preference to colloidal gold? Why was it felt necessary to enhance with silver the 10 nm colloidal gold, which is well within the resolution of the field emission instrument without silver intensification?
E. de Harven: Why was it necessary to silver enhance these 10 nm gold markers so much, apparently about 30 times? Using a Hitachi S-800 FE instrument, 10 nm gold particles can be resolved almost without any enhancement.
Author: Cytocentrifuge preparations, as used here, were originally developed for light microscopy, and the DAB end point could be monitored conveniently by light microscopy, thus provided a quick check on whether any particular procedure had worked. The same principle was also applied when using colloidal gold; in fact, although the gold particles should indeed have been visible in the scanning electron microscope without intensification, the actual number of particles was so low (see Fig. 7f) that it would have been impossibly tedious to locate them without some degree of intensification. It should be emphasised that, at this stage, it was not the intention to demonstrate high resolution, but merely to show that the sensitive antigens being detected could be preserved in chromosomes prepared for scanning electron microscope. High resolution would, of course, be a long term goal.

M. Malecki: Considering the limits in preparation of chromosomes by immunolabelling for SEM which you demonstrated in your paper, and taking into account progress in specimen preparation, including multiple labelling and energy transfers, for laser scanning fluorescence microscopy, would you consider it possible to have some of your questions concerning distribution of the AC1 antibody on chromosomes to be answered by this latter technique?
Author: Confocal laser scanning microscopy would indeed be a potential alternative method for studying the three-dimensional distribution of antibody AC1 (and indeed any other type of label) on chromosomes, albeit with a reduced ultimate resolution compared with SEM. In practice there might be two possible problems. The first concerns the resolution of the microscope in the Z direction, which according to some reports would be scarcely adequate (e.g. Brakenhoff *et al.*, 1989). However, the fact that the distribution of AC1 sometimes appears as a ring round the centromeric constriction by focusing the fluorescence microscope up and down suggests that axial resolution should not, in fact, be a problem. The second point concerns the three-dimensional preservation of the specimen. This is easy to assess using SEM, but not so easy using confocal microscopy, although the latter has the advantage that the specimen can be examined wet.

Additional Reference

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