

2-16-1993

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O. H. Sanchez-Sweatman
McMaster University

E. P. de Harven
University of Toronto

I. D. Dubé
University of Toronto

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Sanchez-Sweatman, O. H.; de Harven, E. P.; and Dubé, I. D. (1993) "Human Chromosomes: Evaluation of Processing Techniques for Scanning Electron Microscopy," *Scanning Microscopy*. Vol. 7 : No. 1 , Article 11.

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HUMAN CHROMOSOMES: EVALUATION OF PROCESSING TECHNIQUES FOR SCANNING ELECTRON MICROSCOPY

O.H. Sanchez-Sweatman¹, E.P. de Harven*, and I.D. Dubé
Department of Pathology, University of Toronto, Toronto, Canada

¹Present Address: McMaster Univ., HSC-3N11, Hamilton, Ont., Canada L8N 3Z5

(Received for publication October 19, 1992, and in revised form February 16, 1993)

Abstract

Methods for scanning electron microscopy (SEM) of chromosomes have been developed in the last two decades. Technical limitations in the study of human chromosomes, however, have hindered the routine use of SEM in clinical and experimental human cytogenetics. We compared different methodologies, including metal impregnation, air drying and specimen coating. SEM preparation of human chromosomes in which osmium impregnation is mediated by tannic acid, yielded more reproducible results when compared with osmium impregnation protocols previously described. The level of osmium impregnation was systematically evaluated by imaging chromosomes in the backscattering mode. Critical point drying and a light gold-palladium coating were essential for appropriate secondary electron imaging of chromosomes. With this method, and in a preliminary quantitative analysis, we show that our SEM technique is more sensitive than light microscopy for the detection of aphidicolin-induced fragile sites. This technical approach is useful for chromosomal studies requiring resolution higher than that obtained by light microscopy. Also, it allows the use of clinical and archival chromosomal samples prepared by routine cytogenetic techniques.

Key Words: Tannic acid, thiocarbonylhydrazide, osmium tetroxide, fragile sites, aphidicolin, cytogenetics.

Introduction

Scanning electron microscopy (SEM) of chromosomes was first reported by Christenhusz *et al.* (1967), as a novel approach to study chromosome ultrastructure. Initial protocols, however, yielded samples with poor preservation of surface details (Neurath *et al.*, 1967; Kingsley Smith, 1970; Tanaka *et al.*, 1970; Iino, 1975). During the last decade, techniques of thiocarbonylhydrazide (TCH)-mediated osmium impregnation have been described that result in remarkably improved SEM images of normal and abnormal chromosomes (Harrison *et al.*, 1981, 1982, 1987; Mullinger and Johnson, 1987; Allen *et al.*, 1988; Niiro and Seed, 1988; Sumner, 1991). Unfortunately the reproducibility of these methods is not completely satisfactory, since differences in appearance between chromosomes of the same preparation have been reported (Allen *et al.*, 1985; Sumner and Ross, 1989). Thus, the thiocarbonylhydrazide ligand does not seem to provide rigorously uniform osmium deposition on each chromatid, making apparent the need for a more reproducible method.

We report herewith our results with an alternate ligand, tannic acid (TA), which has been recommended as a "non-specific ligand" for osmium tetroxide (Simionescu and Simionescu, 1976). We used the elemental contrast provided by the backscattered electron imaging mode of the SEM to assess the intensity of the osmium impregnation of chromosomes. In our hands, tannic acid gave more uniform results enabling us to study, with the SEM, normal and atypical chromosomes. This was particularly useful in our preliminary SEM observations of aphidicolin-induced fragile sites.

The Classic Method

Chromosome preparation

Human chromosomes were obtained from peripheral blood lymphocytes by routine cytogenetic techniques (Watt and Stephen, 1986). In brief, peripheral blood was drawn from a healthy male adult donor and mononuclear cells were cultured in a 5% CO₂/air atmosphere for 72 hours in RPMI-1640 medium (GIBCO Laboratories,

*Address for correspondence:

Etienne de Harven
Department of Pathology
University of Toronto
Banting Institute
100 College Street
Toronto, Ontario
Canada M5G 1L5

Phone No.: (416) 978-2549

FAX No.: (416) 978-7361

New York, NY), supplemented with 20% fetal bovine serum, 2.7 mg/ml L-glutamine, 43.5 U/ml heparin (Hepalean, Organon Teknika, Toronto, ON), 400 U/ml of penicillin and 400 µg/ml of streptomycin at 37°C. Phytohaemagglutinin, 5-10 µg/ml (PHA, Wellcome Diagnostics, Temple Hill, England) was used as mitogen. Metaphase arrest with 0.2 µg/ml Colcemid™ solution (GIBCO Laboratories, New York, NY) for 30 minutes at 37°C, and cell swelling with 75 mM potassium chloride (KCl) were performed. After overnight fixation in cold (4°C) methanol:acetic acid (3:1), the cells were rinsed and resuspended in fresh fixative. Clean 12-mm glass coverslips were mounted on wet, ice-chilled glass slides. Fifty microliters of cell suspension were dropped from a height of about 1 cm onto the coverslips, and air dried at room temperature. Metaphase spreading was facilitated by slightly tilting the coverslips while drying. Specimen quality was initially assessed under the phase contrast microscope. If necessary, chromosome solid-staining was achieved by immersing the coverslips for 2 minutes into 10% Leishman's stain (BDH Chemicals, Toronto, ON) in Gurr's buffer.

Thiocarbohydrazide-Mediated Osmium Impregnation

The protocol of Harrison *et al.* (1987) was followed. Dry TCH (Polysciences, Warrington, PA), was kept in a desiccator, in the dark at 4°C. Immediately before use, it was diluted to a 1-2% concentration in distilled water at 60°C. After cooling to room temperature, the saturated solution was filtered through 0.22 µm filters (Millex-GS, Millipore, Bedford, MA). Air dried cells were fixed with 2.5% glutaraldehyde (J.B.E.M. Services, Quebec) in Sorensen's buffer, pH 7.4, for 30 minutes at room temperature. Rinsing in buffer and fixation with 1% osmium tetroxide (OsO₄) in Sorensen's buffer for 10 minutes was followed by three rinses of 2 minutes each with distilled water. Specimens were then incubated with TCH for 10 minutes, rinsed 3 times with distilled water and immersed into 1% OsO₄ in distilled water for 10 minutes. The TCH-OsO₄ treatment was repeated once after distilled water rinses as described above. Ten minute dehydration steps through a series of 50%-100% ethanol preceded critical point drying (Polaron, Watford, England) from bone dry carbon dioxide. Dried samples were glued to aluminum stubs and sputter-coated with gold or gold-palladium (Denton Vacuum Desk-1 Cold Sputter-Etch Unit, Denton Vacuum, Cherry Hill, NJ) for 20-30 seconds, in a residual argon atmosphere of 75 millitorr and a direct current of 40 milliamperes.

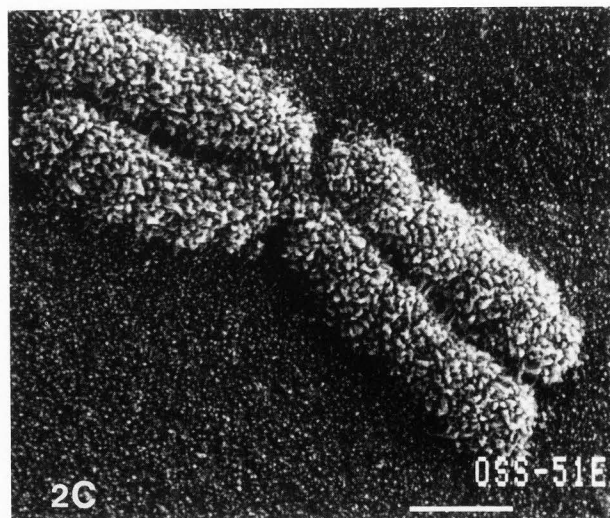
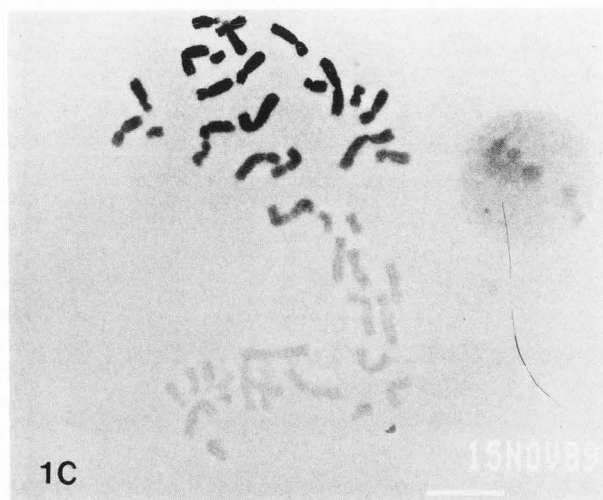
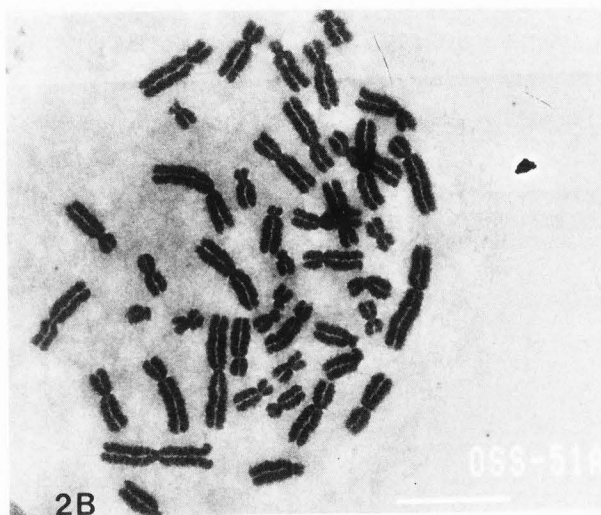
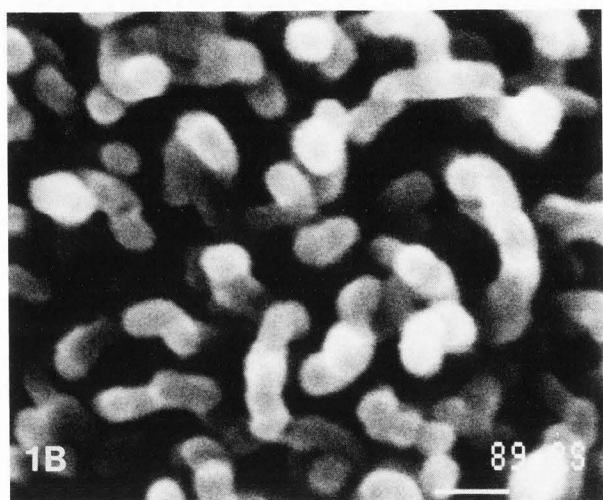
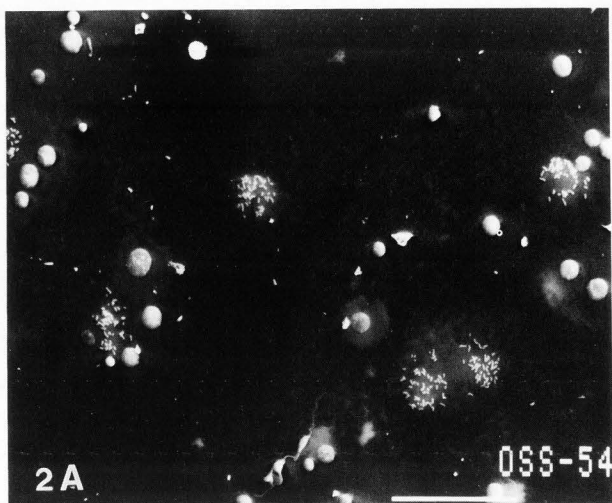
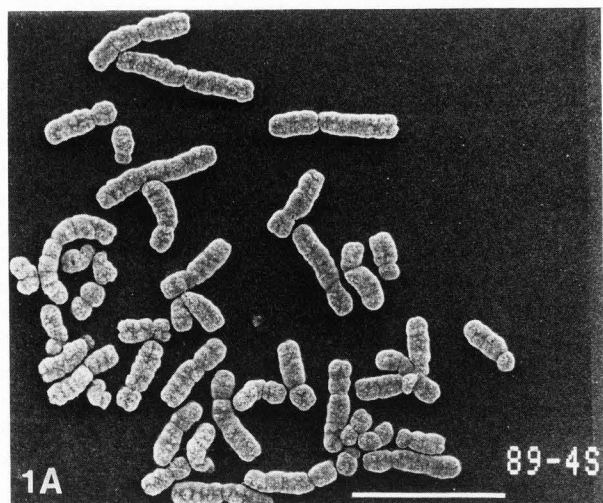
Most chromosomes prepared with the OsO₄-TCH protocol generated an intense secondary electron signal and displayed well preserved morphology (Figure 1A). At high magnification, centromeres and chromatids were readily identified and a well defined fibrillar surface was evident (Figure 1B). The fibrillar structures displayed diameters of 50-70 nm. These dimensions agree with those reported by others (Adolph, 1988), and are interpreted as consistent with 30-nm chromatin fibers covered by a 10-20 nm layer of osmium and heavy metal coating.

Figure 1 (facing page, left). Scanning electron micrographs of human metaphase chromosomes prepared with thiocarbohydrazide-mediated osmium impregnation. (A) The specimen was air dried, exposed to trypsin, fixed in glutaraldehyde, impregnated with OsO₄-TCH, dehydrated, critical point dried and gold-sputter coated. Chromosomal structure is clearly defined and chromatid segmentation is observed. Working distance = 8 mm, 20 kV, bar = 10 µm. (B) Specimen prepared with thiocarbohydrazide-mediated osmium impregnation as above, but omitting trypsin treatment. The chromosomal surface reveals the presence of fibers with a diameter of 50-70 nm, some of which formed "loops". Working distance = 8 mm, 20 kV, bar = 100 nm. (C) Backscattered electron imaging demonstrates uneven osmium impregnation of chromosomes within a metaphase spread. Chromosomes in the upper half of the figure are better impregnated. Working distance = 14 mm, 20 kV, bar = 10 µm.

Figure 2 (facing page, right). Scanning electron micrographs of human metaphase chromosomes prepared with tannic acid-mediated osmium impregnation. (A) The specimen was air dried, fixed with glutaraldehyde, impregnated with OsO₄-TA, dehydrated, critical point dried and gold-sputter coated. Strong and uniform topographical contrast was evident in 5 adjacent mitotic spreads and in several interphase nuclei. Working distance = 14 mm, 20 kV, bar = 100 µm. (B) Backscattered electron imaging demonstrates even osmication of chromosomes within a metaphase spread. Working distance = 8 mm, 20 kV, bar = 10 µm. (C) Optimal chromosomal morphology and structural detail is observed in this metacentric chromosome. Working distance = 14 mm, tilt angle = 20 degrees, 20 kV, bar = 1 µm.

Having a large experience in the elemental contrast provided by the backscattered electron imaging (BEI) mode of the SEM (de Harven and Soligo, 1989), we took advantage of the remarkably efficient separation of the secondary electron and backscattered electron signals consistently provided by the JEOL-JSM 840 scanning electron microscope. This instrument was equipped with a lanthanum hexaboride (LaB₆) cathode and operated at a vacuum of approximately 3×10^{-7} torr, under 15-20 kV accelerating voltage. The elemental contrast of osmium (atomic number, $Z = 76$), observed in the backscattered mode of the SEM, clearly revealed marked differences between the intensity of osmium impregnation of chromosomes of the same metaphase (Figure 1C). The elemental contrast generated by the sputtered gold ($Z = 79$) conductive coating was presumably uniform. Differences in the intensity of the BEI signal were, therefore, interpreted as originating from differences in the degree of osmium deposition. Such differences were repeatedly observed in several experiments. Attempts to

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alleviate the problem were made by modifying the technique in different steps of the preparation procedure and included: (a) fixatives, such as paraformaldehyde and ethanol; (b) rinsing buffers, such as phosphate-buffered saline and Tris-HCl; (c) increasing number and duration of cycles of exposure to OsO₄-TCH; (d) drying procedures, such as air drying and Peldri II (Kennedy *et al.*, 1989); and (e) carbon coating or no coating at all (see below). It soon became clear, however, that these modifications of the technique were unable to secure even osmium impregnation of all the chromosomes of any given metaphase spread. We then substituted thiocarbonylhydrazide with another osmium ligand, tannic acid.

The Tannic Acid Method

Tannic Acid-Mediated Osmium Impregnation

Tannic acid was tested as ligand for osmium tetroxide. Air dried coverslips were fixed with 3% glutaraldehyde in Sorensen's buffer, pH 7.4, for 30 minutes at room temperature. After 3 buffer rinses, 1% OsO₄ in Sorensen's buffer was added onto the samples and left for 10 minutes, followed by 3 rinses in double distilled water. Specimens were then incubated with freshly prepared, filtered 2% TA (Tannic Acid AR, Mallinckrodt, Paris, KY) for 10 minutes. Coverslips were rinsed 3 times in distilled water and treated with 1% OsO₄ in distilled water for 10 minutes. Treatments with TA and OsO₄ were repeated once. Ethanol dehydration, critical point drying and sputter coating with gold were performed as described above.

The tannic acid based method provided uniform osmium impregnation of all the observed chromosomes. Figure 2A, taken at very low magnification, shows five adjacent metaphases in the same secondary electron imaging (SEI) contrast. We could never achieve such uniformity with the TCH-based method. We assessed the level of osmium impregnation in the elemental contrast of the BEI mode and confirmed that all chromosomes emitted BEI signals of identical intensity (Figure 2B). Various protocols of TA-mediated OsO₄ impregnation were tested. Phosphate-buffered saline, Sorensen's buffer pH 7.4, Tris-HCl pH 7.5, and distilled water were compared as diluents. The best preservation was demonstrated in chromosomes treated with OsO₄-TA-OsO₄-TA-OsO₄ (Figure 2C). Sorensen's buffer in the first osmium treatment was also required for optimal structural preservation.

The possibility that TA-mediated osmication of chromosomes could induce more pronounced shrinkage than the TCH-mediated method (Murphy, 1978; Murakami and Jones, 1980) was studied by measuring the length of the 10 longest chromosomes in 10 metaphases from specimens prepared by the two methods. No significant difference ($p = 0.2752$) was observed between the two groups of measurements (total averages: $9.46 \pm 1.17 \mu\text{m}$ for TCH, and $8.83 \pm 1.33 \mu\text{m}$ for TA).

Figure 3 (facing page, left). Scanning electron micrographs of uncoated human metaphase chromosomes spread on conductive coverslips. (A) Chromosomes were spread on carbon-coated glass coverslips, air dried, fixed with glutaraldehyde, impregnated with OsO₄-TA, dehydrated and critical point dried. No coating was used. Samples are characterized by a poor SEI signal contrast, with morphologically preserved chromosomes. Working distance = 15 mm, tilt angle = 20 degrees, 20 kV, bar = 10 μm . (B) An uncoated chromosome displays poor surface detail. Working distance = 14 mm, tilt angle = 20 degrees, 20 kV, bar = 1 μm . (C) Gold-sputter coating was performed on the same chromosome sample as in figure 3B. Surface detail which was not resolved on uncoated samples, is now visualized. Working distance = 14 mm, tilt angle = 20 degrees, 20 kV, bar = 1 μm .

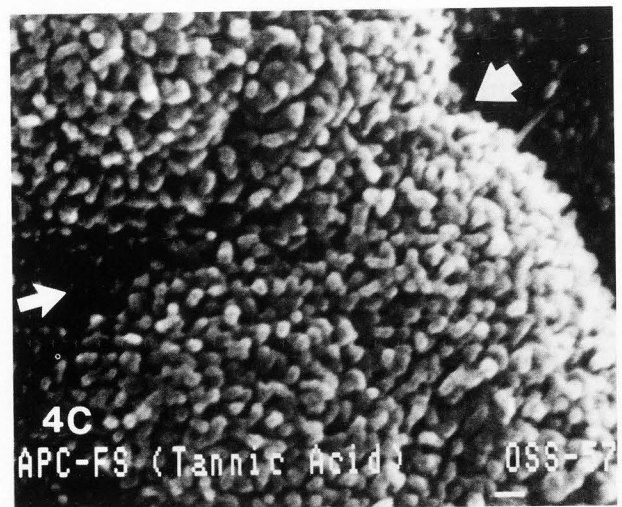
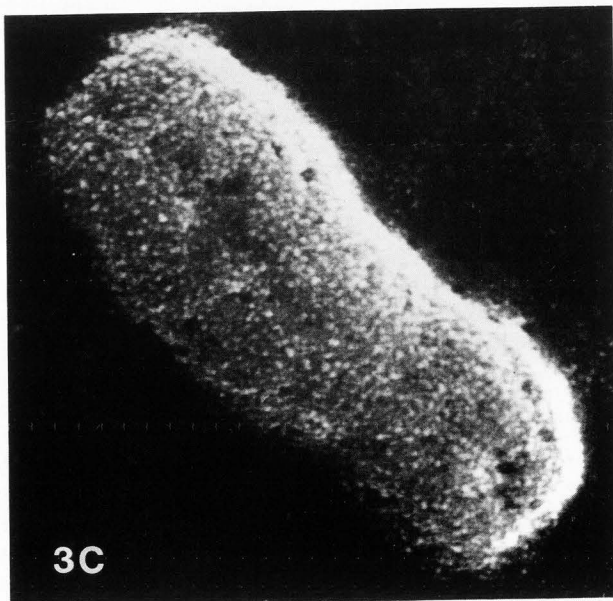
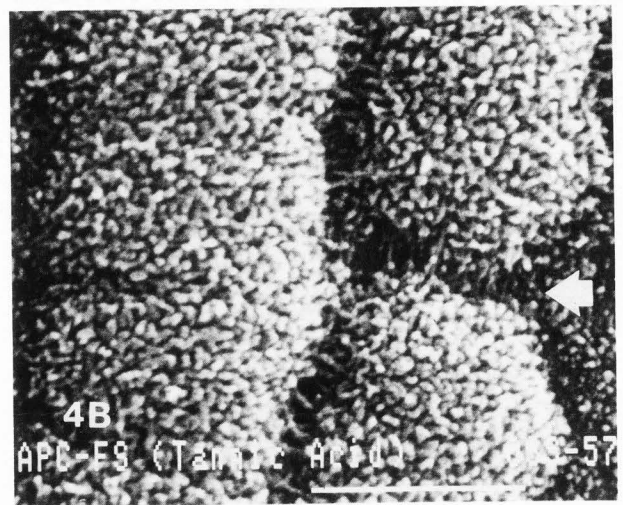
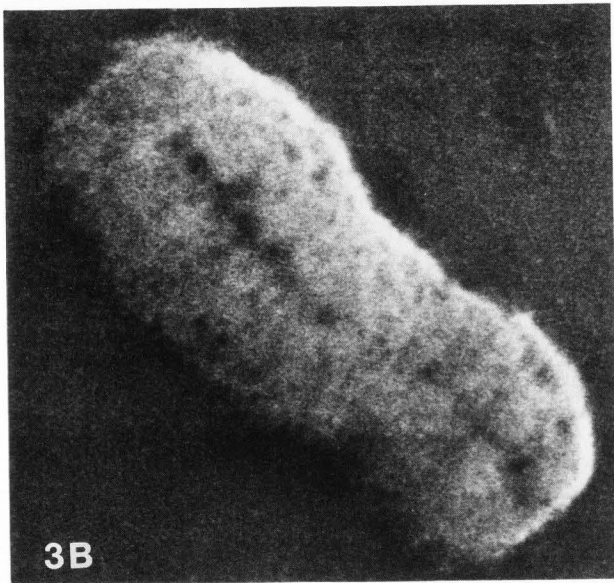
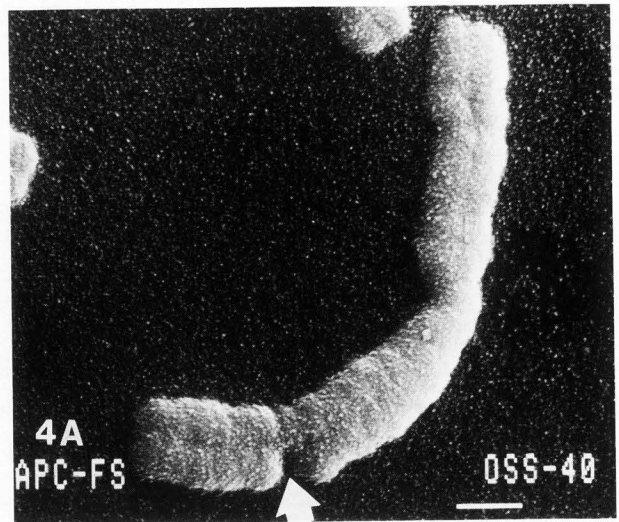
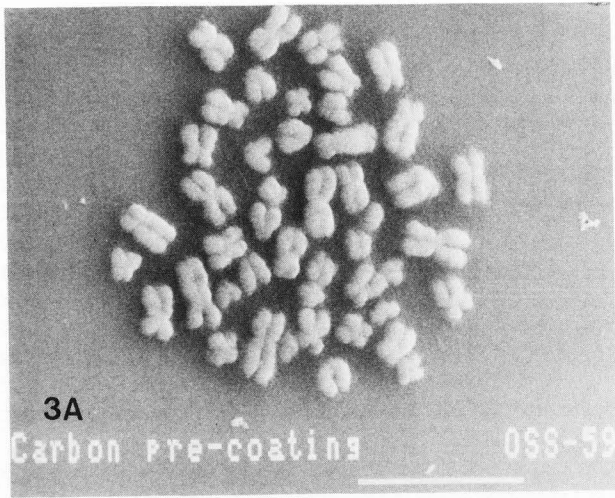
Figure 4 (facing page, right). Scanning electron micrographs of aphidicolin induced fragile sites on human metaphase chromosomes. (A) Cultured lymphocytes were exposed to APC for 24 hours before harvesting. Sample was prepared with OsO₄-TCH protocol. A chromatid gap involving one of the long arms is observed (arrow). Working distance = 15 mm, tilt angle = 20 degrees, 20 kV, bar = 1 μm . (B) A gap involving one chromatid is observed (arrow). Few fibers link the proximal and distal segments on the affected chromatid. In contrast, many fibers still connect these fragments with the paired chromatid. Working distance = 14 mm, tilt angle = 20 degrees, 20 kV, bar = 1 μm . (C) The "unaffected" chromatid (large arrow), frequently showed a groove at the same location as the FS in the paired chromatid (small arrow). Working distance = 14 mm, tilt angle = 20 degrees, 20 kV, bar = 100 nm.

Attempts to Minimize Conductive Coating

Techniques of TCH-mediated osmication were classically reported as yielding samples adequately conducting as a result of metallic osmium deposition (Murphy, 1978). Theoretically, the imaging of heavily osmicated chromosomes should not be impeded by charging artifacts. In fact, however, this is not the case: electrostatic charging was a consistent problem in all our samples.

We reasoned that charging resulted probably more from the non-conductive glass coverslip substrate used in all our preparations than from the poor conductivity of the osmicated chromosomes. To put this hypothesis to a test, glass coverslips were heavily carbon coated before being used for metaphase spreading. The TA method was applied to these samples which, interestingly, were practically free of charging (Figure 3A). However, when one of these samples was observed at higher magnification it became clear that the fibrillar architecture of chromosome surfaces was not recognizable

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(Figure 3B). The sample illustrated in Figure 3B was then taken out of the SEM, sputtered with gold and the very same chromosomes re-photographed, providing this time adequate surface morphology (Figure 3C).

Obviously, the conductive coating procedure contributes greatly to what we tend to regard as the "well preserved" surface morphology of chromosomes. It remains likely, however, that heavy carbon pre-coating of the glass substrate will permit to minimize the thickness of conductive coating of chromosomes in future experiments.

Different Drying Procedures

To compare the ultrastructure of chromosomes dried by critical point drying (CPD) or with "Peldri II" (Ted Pella, Redding, CA), other specimens were dried with the latter, according to a method previously described (Kennedy *et al.*, 1989). After osmium impregnation and ethanol dehydration, coverslips were immersed in warm (40°C) 1:1 Peldri II/ethanol solution for 45 minutes. The specimens were then transferred to warm 100% Peldri II for another 45 minutes, after which time they were placed on ice until complete solidification of the Peldri II. Sublimation of the Peldri II was achieved under vacuum at room temperature, under environmentally safe conditions which permitted the total solid phase recovery of the Peldri II.

Chromosomes dried with CPD or the Peldri II procedure displayed very similar levels of ultrastructural preservation. Air dried controls revealed drastically damaged ultrastructure, as anticipated.

Application of the Tannic Acid Method to the Enumeration of Fragile Sites

Convinced about the apparent inevitability of osmium and of gold sputtering coating, we then hypothesized that the tannic acid method, by being the most effective and reproducible we had found, may perhaps facilitate effective enumeration of induced fragile sites (FS).

Induction of Fragile Sites

Aphidicolin (APC) solution was prepared by diluting lyophilized APC (Sigma, St. Louis, MI) in 0.2% dimethylsulfoxide (DMSO) in distilled water and kept at 4°C. Peripheral blood mononuclear cells were obtained as described above. 2.7×10^4 - 3.5×10^4 cells/ml were incubated in 10 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, penicillin, streptomycin and 10 µg/ml of phytohaemagglutinin at 37°C in a 5% CO₂/air atmosphere. Seventy-two hours later, APC was added to a final concentration of 0.2 µM, according to the method of Glover *et al.* (1984). After a 24 hour incubation, cells were arrested at metaphase with Colcemid, swollen with hypotonic KCl and fixed in methanol:acetic acid as described above. Metaphase spreads were studied with the light microscope (LM) under oil immersion, after Leishman's solid-stain-

ing. For SEM, chromosomes were prepared by the tannic acid method described above.

Under SEM, fragile sites appeared as gaps involving one or both chromatids (Figure 4A), reminiscent of those observed under LM. At high magnifications (over 50,000x), wide chromatid gaps that had few or no fibers connecting distal and proximal segments were observed (Figure 4B). When a FS involved only one chromatid, the unaffected paired chromatid, although not forming a gap, frequently revealed a groove or constriction at the corresponding site (Figure 4C).

To compare the efficiency of fragile site detection with LM *versus* SEM, chromosome spreads from the same samples were prepared for both. Metaphases with one or more gaps, breaks or triradial figures were counted as "positive" for FS. Those in which chromatid non-staining areas were present, but without evidence for gap formation, were counted as "suggestive". The remaining metaphases were recorded as "negative" for FS. One hundred metaphases were counted in each experiment. Chi-square tests were used to analyze the significance of differences in FS counts.

The number of metaphases containing fragile sites was counted on chromosome spreads originating from the same preparations under LM and SEM. Under the LM, aphidicolin-induced FS were observed in $8.5 \pm 1.5\%$ of metaphases. Additionally, $18.5 \pm 6.5\%$ of metaphases showed images suggestive of their presence. As seen in Table 1, a significantly higher number of FS-positive metaphases (20.0 ± 2.0) was observed under the SEM.

Discussion

Our initial aim was to quantify induced fragile sites more effectively than currently achieved with the light microscope. Scanning electron microscopy appeared as offering an attractive approach because its resolution is considerably higher than that of the light microscope. Of course, transmission electron microscopy offers even higher resolution. Unfortunately it remains extremely difficult to view many whole metaphases under the TEM, making quantitative studies practically impossible.

Success in quantifying fragile sites by scanning electron microscopy necessitates a technique yielding uniform topographical contrast on all the chromosomes of any given preparation of mitotic spreads. Unfortunately, the techniques based on the use of the TCH ligand for osmication of chromosomes do not, in our hands and in those of others (Allen *et al.*, 1985; Sumner and Ross, 1989), offer the desirable uniformity of topographical contrast. This is somewhat surprising since TCH-mediated osmium impregnation gave apparently satisfactory imaging on non-chromosomal biological samples (Kelley *et al.*, 1973; Ip and Fischman, 1979). The limitations of TCH-mediated osmium impregnation were analyzed by Sumner and Ross (1989). After studying each step in the OsO₄-TCH protocol, these authors

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demonstrated that this procedure removes parts of the chromosomal surface, thus revealing internal structures. The variability found on chromosomal osmication could then perhaps be explained by removal of layers of cytoplasmic debris and superficial non-histone nucleoproteins, as a non-specific uncontrolled process.

We have demonstrated that the intensity and uniformity of the osmium impregnation of chromosomes can be readily assessed by observing the elemental contrast in the backscattered electron imaging mode of the SEM. This, however, requires effective separation of the SEI and BEI signals. Such signal separation is easily obtained with the SEM used in the present study, but is apparently not satisfactorily achieved with instruments from other manufacturers. Backscattered electron imaging of chromosomes prepared by the TCH-osmium protocols demonstrated a significant lack of uniformity in the level of osmium impregnation. At variance, when the tannic acid ligand was used, uniform levels of osmium impregnation were reproducibly demonstrated. This correlated with a very uniform topographical contrast on all the chromosomes prepared by this method. The TA-OsO₄ method appears, therefore, more reliable in quantitative studies, as indicated by our preliminary enumeration of aphidicolin-induced fragile sites. Under the SEM, the counted numbers of FS were significantly higher than those observed under the light microscope.

Fragile sites are specific areas on chromosomes at which gaps or breaks occur non-randomly in a low percentage of cells under conditions of thymidylate stress. Interest in their study has increased due to the association of a rare FS on Xq27.3 with a common inherited mental retardation syndrome (Sutherland and Hecht, 1985). Moreover, an association with some site-specific cytogenetic abnormalities in neoplastic cells has been hypothesized (Hecht and Sandberg, 1988). It is generally agreed that they represent chromosomal regions in which chromatin fibers are not properly condensed (Nussbaum and Ledbetter, 1986). Chromosomal fragile sites were first demonstrated under SEM by Harrison *et al.* (1983). These authors accurately determined the location of the fragile site on the X chromosome, associated with the X-linked mental retardation syndrome (Martin-Bell Syndrome). However, no information on autosomal FS was reported.

Tannic acid was originally introduced as an additional "fixative" for electron microscopy of biological specimens (Mizuhira and Futaesaku, 1971). It enhances osmication of biological specimens, allowing observation of uncoated samples with the SEM (Sweny and Shapiro, 1977; Murakami and Jones, 1980). For chromosome studies, Sweny *et al.* (1979) and more recently Naguro *et al.* (1990) used TA-OsO₄ to visualize uncoated chromosomes. With human chromosomes, however, a light coating was required for imaging at magnifications over 20,000x. We consider the TA method as superior to the TCH method because of the uniformity of the results, not because of any advantage in resolution or visualization of fine structural details.

Table 1. Number of fragile sites induced by aphidicolin and detected by light microscopy and scanning electron microscopy (%)*.

	POSITIVE	SUGGESTIVE	NEGATIVE
LM	8.5 ± 1.5	18.5 ± 6.5	73.0 ± 5.0
SEM	20.0 ± 2.0	15.0 ± 4.0	65.0 ± 6.0

*Chromosome spreads from the same samples were prepared for both LM and SEM. One hundred metaphases were counted in each sample studied. A higher number of FS-positive metaphases was observed under SEM ($p = 0.013$). No fragile sites were observed in control samples exposed to DMSO (APC vehicle) or those not exposed to increasing concentrations of aphidicolin (data not shown).

The persisting limitation of the TA method resides, however, in its dependency on conductive coating to visualize the fibrillar architecture of chromosome surfaces. Of concern is the question of the inherent difficulty to visualize, in the BEI mode of the SEM, small colloidal gold markers which could be used in further studies. Allen *et al.* (1985), however, demonstrated in the BEI mode the strong signal generated by silver deposition on metaphase chromosomes. Obviously, much higher magnifications would be required to visualize small colloidal gold markers. At such high magnifications, it is anticipated that the elemental contrast of the conductive coating will most likely obliterate that of 5 or 10-nm gold particles. Minimizing the thickness of the coating and/or substituting gold with chromium will probably make the imaging of such small markers possible and, therefore, open the way for interesting new studies which could include the localization of specific DNA sequences by *in situ* hybridization methods.

Acknowledgments

Dr. O.H. Sanchez-Sweatman performed this work while being the recipient of a University of Toronto's Open Master's Fellowship and an Ontario Graduate Scholarship from the Ministry of Colleges and Universities of the Province of Ontario. The financial support of the Medical Research Council of Canada and the Leukaemia Research Fund, through operating research grants to Dr. Etienne de Harven and Dr. Ian Dubé is gratefully acknowledged.

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Discussion with Reviewers

A.T. Sumner: Many procedures for preparing chromosomes for SEM incorporate a light trypsin treatment before glutaraldehyde fixation. Have you tried this, and might lack of it explain the variability of your results with TCH, and lack of detailed surface structure (e.g., with uranyl acetate, or without coating)?

Authors: We treated metaphase spreads with trypsin for 20-300 seconds and immediately thereafter fixed them in glutaraldehyde followed by TCH or TA preparation for SEM. Transversal chromatid indentations or "grooves" were observed in patterns corresponding to G bands observed on Giemsa-stained spreads under light microscopy. However, we did not observe any improvements in the quality of TCH-treated preparations with this treatment. Trypsin-induced effects on chromosome ultrastructure have been discussed in detail by Allen *et al.* (1988).

A.T. Sumner: Have you attempted to use a low accelerating voltage to eliminate charging on uncoated specimens?

Authors: Yes, we have. Although the decrease of the accelerating voltage with uncoated osmium-impregnated chromosome spreads reduced the overall charging, it severely affected resolution below 5 kV. We concluded that 20 kV accelerating voltage was most favorable for optimum imaging under the conditions of our observations.

A.T. Sumner: Have you used any conductive substrates other than carbon-coated glass, and are there any problems with spreading chromosomes on conductive substrates?

Authors: We tested carbon and gold/palladium as conductive substrates. Although both greatly reduced electrostatic charging, we observed that metaphase spreading was inadequate on gold/palladium-coated coverslips. In contrast, carbon coating did not affect spreading.

A.T. Sumner: Since you obtained little or no detailed surface structure without coating, is it possible that some of the surface structure is an artefact produced by coating?

Authors: Our interpretation of these observations is that coating is required for adequate imaging of chromosomal surface details. This notion is confirmed by the fact that most workers in this field use coated samples. As with all coated biological structures, the possibility of decoration artifacts cannot be ruled out.

A.T. Sumner: Apart from the increased rate of detection of fragile sites, have your studies with SEM provided any new insights into the nature of fragile sites?

Authors: We observed that scanning electron micrographs of chromosomes from aphidicolin-treated lymphocytes not only displayed wide typical chromatid gaps, but also narrower gaps and/or grooves affecting one or

both chromatids. These more subtle lesions were not observed on light microscopy. These observations lead us to believe that the chromatid gaps observed under light microscopy are only the end of a spectrum of aphidicolin-induced defects ranging from minor chromatid indentations to gaps and/or deletions characteristic of fragile sites.

Hans Ris: It is well known that air drying severely damages cell structures due to surface tension forces. The authors stress that after conductive staining the chromosomes must be dried either by the critical point drying method or by the Peldri II technique to preserve their ultrastructure. In the classic method for spreading the arrested metaphases, cell suspensions in methanol: acetic acid fixative are dropped on coverslips, **air dried**, and then fixed in glutaraldehyde. How do you explain that the fibrillar ultrastructure of chromosomes survives the air drying at this stage of the procedure?

Authors: Our experiments did not directly address the effects of air drying on chromosomal ultrastructure and were all performed after air drying since we aimed to apply our technology to routine clinical cytogenetic samples. However, Allen *et al.* (1985) compared the ultrastructure of non-air-dried chemically isolated chromosomes with air dried metaphase spreads without noticeable differences upon SEM imaging. We speculate that nucleic acids may have an inherent resistance to damage by air drying, in contrast with lipid-rich cellular and subcellular membranes.

Hans Ris: How does methanol-acetic acid fixation affect the native chromosome structure, or alter the chemical composition?

Authors: Many investigators in the field share similar concerns regarding the "harsh" fixation and spreading methods necessary to obtain adequate chromosome preparations. As described in the text, we evaluated other fixatives, but, not surprisingly, found that methanol: acetic acid is required for preserving the chromosomal structure in a way suitable for cytogenetic analysis. This fixative is known to extract histone and non-histone proteins, without affecting the chromosomal DNA [Burkholder (1988). In: Chromosome structure and function. Gustafson JP, Appels R (eds.), Plenum Press, NY, pp 20).

T.D. Allen: What do you estimate to be the thickness of the coating applied to the chromosomes?

Authors: Although we did not measure this parameter, we can deduce that the thickness of the coating layers (osmium and heavy metal) is of the order of 20-30 nm. We base this estimate on the accepted 30-nm diameter of chromatin fibres in metaphase chromosomes and the diameters observed by us of 50-70 nm (see Figure 1B).

T.D. Allen: The criticism of TCH as an osmium impregnation vehicle appears to be mainly one of inconsistency rather than absolute retention of fine structure and

generation of secondary electron signal. Can the authors confirm that on the best areas of their OsO₄-TCH preparations, there was as good structural preservation and SE signal generation as OsO₄-TA preparations?

Authors: Indeed, surface detail in the osmium-impregnated areas of chromosome spreads treated with OsO₄-TCH was superb, as depicted in Figure 1B. As well, perusal of the literature provides remarkable examples of good quality imaging using this protocol. However, to our knowledge, none of the previous studies addressed the issue of uniformity throughout the preparation. In our work, this was an obvious limitation as soon as we attempted to quantify aphidicolin-induced fragile sites in the same preparation.