Scanning Electron Microscopy

Volume 1985 | Number 3 Article 22

7-12-1985

A Scanning Electron Microscopic Study of Chromosomes and Nuclei

Kazuhiko R. Utsumi
Aichi Cancer Center Research Institute, Nagoya

SCANNING ELECTRON MICROSCOPY/1985/III (Pages 1121-1132)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507 USA

0586-5581/85$1.00+.05

A SCANNING ELECTRON MICROSCOPIC STUDY OF CHROMOSOMES AND NUCLEI

Kazuhiko R. Utsumi*

Laboratory of Cell Biology
Aichi Cancer Center Research Institute
Nagoya, Japan

(Paper received January 29, 1985: manuscript received July 12, 1985)

Abstract

The fine structure of metaphase chromosomes and nuclei were studied by scanning electron microscopy. A coiled-coil structure of chromosomes was suggested by hypotonically unravelled chromosomes observed under light microscope. But chromosome preparations made for the light microscopic studies were not adequate for detailed examination with scanning electron microscope. Surface-spread chromosomes revealed that they were composed of nodular, twisted looping fibers of about 300 Å in diameter. Surface-spread nuclei were also composed of fibers identical to the chromosome fibers.

KEY WORDS: Chromosome fibers, nuclear fibers, structure of chromosomes, structure of nuclei, sputter-coating, decoration effects, chromosome uncoiling, "footprints" of chromosomes, stereography, surface-spreading technique.

*Address for Correspondence:
Laboratory of Cell Biology, Aichi Cancer Center Research Institute, 81-1159 Kanokoden, Chikusa-ku, Nagoya 464, Japan Phone No. (052) 762-6111

Introduction

The spiral structure of chromosomes of Tradescantia pollen mother cells was first reported by Baranetzky (1880) more than a hundred years ago. Kuwada (1939) summarized and discussed the mechanism of chromosome spiralization. Based on the observations with light microscopy (LM), many theories and explanations of chromosomal structure have been proposed without presenting any positive evidence. The limitation of resolution of the LM impeded the study of chromosomal fine structure. Technical development and practical use of electron microscopy, however, has led to rapid progress in the study of chromosomal fine structure.

Transmission electron microscopy (TEM) offers high resolution, but also has the limitation that the meterial to be examined should be ultrathin and put on a grid 3 mm in diameter. Burkholder (1971) observed trypsin-banded chromosomes which were spread on a carbon-enforced Formvar film floated on water. Gormley and Ross (1972) observed C-banded chromosomes made by the ASG technique (Sumner et al., 1971) using a carbon replica which was shadowed with gold-palladium after floating off the slide. Ruzicka (1974) coated chromosomes with carbon and the resulting carbon film and chromosomes were floated off the slide into 0.3 N hydrofluoric acid. Burkholder (1981) prepared first R-banded chromosomes on a plastic coated microscope slide, then the film was foated off the slide into the water. Xu and Wu (1983) coated G-banded chromosomes with Parlodion film which was then floated off the slide into 0.06 M phosphate buffer or 0.1 M hydrofluoric acid. In these studies, the floating film to which chromosomes were attached was picked up onto an EM grid.

Gall (1963, 1966) spread the newt erythrocyte nuclei or human chromosomes directly on the water surface. The surface-spread material was picked up on carbon-coated EM grids, then critical point-dried (Anderson, 1951), and shadowed with platinum-palladium. DuPraw (1966) proposed a folded-fiber theory of chromosome structure after intensive studies using this technique. Although this technique revealed that chromosomes were composed of fibers, chromosomes were too thick for the detailed examination by TEM and the

higher-order arrangement of the fiber was still obscured. High voltage TEM (Nakanishi et al., 1970) and/or stereography (Stubblefield and Wray, 1971; Ris and Witt, 1981; Mullinger and Johnson, 1983) was able to overcome this difficulty.

The elucidation of substructure of chromatin fibers such as beads-on-a-string structure (Olins and Olins, 1974) and solenoidal model (Finch and Klug, 1976), however, was significant contribution made using TEM.

Scanning electron microscopy (SEM) is suited for the analyses of the higher-order structure of chromosomes, because the available images are three-dimensional (Golomb, 1976). Findings of the spiral structure of chromosomes by treatment with hypotonic solutions (Utsumi and Tanaka, 1975; Utsumi,1976) has led me to further investigations of higher-order structure of chromosomes using SEM.

This paper reviews the TEM and SEM studies on chromosomes prepared using different techniques, but does not include those on plant chromosomes (Laane et al., 1977), amphibian lampbrush chromosomes (Angelier et al., 1984), or polytene chromosomes (Meyer and Lipps, 1984). New results about chromosome fibers and nuclear fibers revealed by SEM in the surface-spread cells are also presented.

Uncoiling of Chromosomes

In animal cells, a spiral chromosome structure comparable to that of plant cells was not clearly exhibited until Ohnuki (1968) demonstrated it in human lymphocyte chromosomes. Utsumi then found that the uncoiling of chromosomes was obtainable merely by a selection of hypotonic solutions used for the swelling of cells (Utsumi and Tanaka, 1975; Utsumi, 1976). The uncoiling effect of hypotonic solutions of sodium citrate on chromosomes was studied on Raji cells. Hypotonic solutions of a series of doubling dilutions of isotonic solution of sodium citrate from 1/2 to 1/32 were tested. Hypotonic treatments were done for 20 min at room temperature with these dilutions, and preparations were made by the ordinary air-drying method. An optimal dilution was decided by the degree of uncoiling of chromosomes. With 1/16 isotonic solution of sodium citrate (for concentration, see Table 1), chromosomes demonstrated a loosely uncoiled structure (Fig. 1). The effectiveness of the hypotonicity on the chromosome uncoiling was able to be estimated by examination of nuclei, because nuclei became homogeneous at concentrations of hypotonic solution which caused chromosomes to uncoil.

Non-electrolytes such as dextrose, fructose, glycerol, and sucrose also had the uncoiling effect as did electrolytes, such as ammonium chloride, calcium chloride, magnesium chloride, potassium acetate, potassium chloride, sodium acetate, sodium chloride, and sodium citrate. Most of the solutes examined had an optimum effect of chromosome uncoiling at 1/4 or 1/8 isotonic solution, whereas glycerol at 1/1.5 and sodium citrate at 1/16. Magnesium chloride and calcium chloride were most effective with 1/8 isotonic solution, but caused rupture of cells with further dilution.

In the slightly uncoiled chromatids, one can count the numbers of gyres (Fig. 1a). The number of gyres in chromatids decrease in proportion to their length or to the degree of condensation occurring during the mitotic phase. In the extremely uncoiled chromatids, one can see a lower-order coil (Figs, 1b, c). The spiral structure of chromosomes, however, was not clearly confirmed using the LM due to the limitation in resolution. Therefore, attempts to elucidate the fine structure of chromosomes prepared to the LM studies were made by SEM.

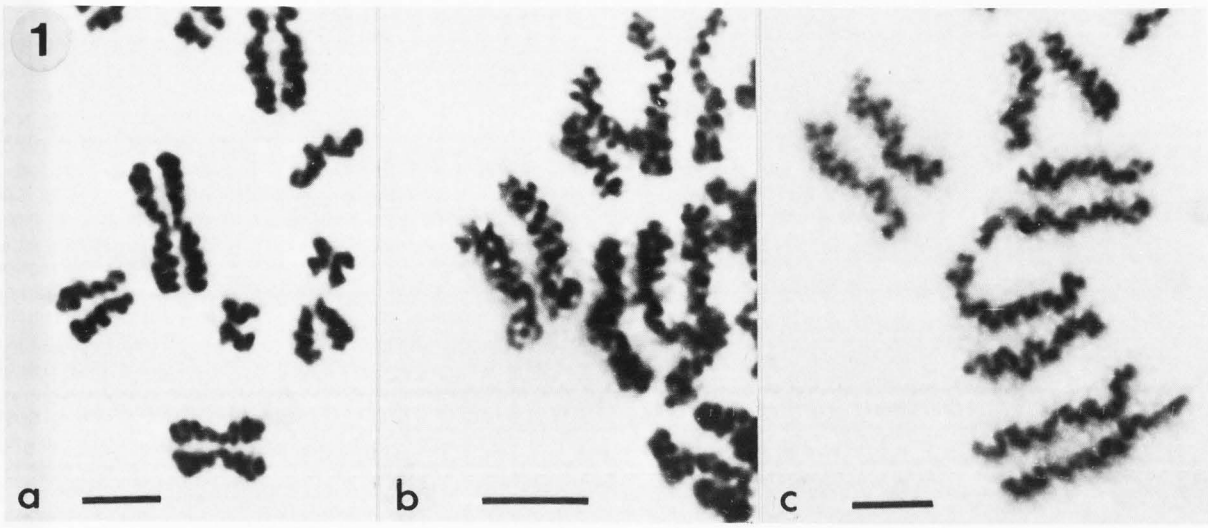
Table 1. Optimum concentrations of hypotonic solutions effective for chromosome uncoiling

	Dilution of isotonic solution	Concentration (mM)
Dextrose	1/4	70.0
Fructose	1/4	70.0
Glycerol	1/1.5	188.0
Sucrose	1/4	67.5
Ammonium chloride	1/8	18.8
Calcium chloride	1/8	14.5
Magnesium chloride	1/8	26.5
Potassium acetate	1/8	19.5
Potassium chloride	1/4	40.0
Sodium acetate	1/8	30.9
Sodium chloride	1/4	38.5
Sodium citrate	1/16	6.4

SEM Studies of LM Chromosomes

SEM was applied to the study of chromosomes soon after its development (for historical review, see Golomb, 1976). Chromosome preparations made for LM had to be coated with heavy metal to avoid charging when examined under SEM. In earlier experiments, LM preparations were coated in a vacuum with a thin layer of evaporated gold (Neurath et al., 1967; Pawlowitzki et al., 1968; Pawlowitzki and Blaschke, 1971; Yu, 1971), or aluminium (Christenhuss et al., 1967). Recently, chromosomes were sputter-coated with gold-palladium (Daskal et al., 1976), or gold (Utsumi, 1981, 1982) in an ion-coater. Coating with heavy metals, however, has the disadvantage that vacuum, ion bombardment and heat produced during coating may produce artifacts such as the masking of superficial fine structure and distortion of soft biological materials. Therefore, the conductive staining such as glutaraldehyde-tannic acid and osmium tetroxide fixation procedure (Sweney et al., 1979) or an osmium tetroxide-thiocarbohydrazide fixation method (Harrison et al., 1981, 1982; Mullinger and Johnson, 1983) may be preferable for uncoated chromosomes.

Selected metaphase chromosomes were marked with an object marker, and they were photographed by LM. These marks on the slide and photographs were used as guides in the SEM examinations. The microscopic slides were cut into small pieces according to the distribution of the marks, and each of them was mounted on an aluminium stub with



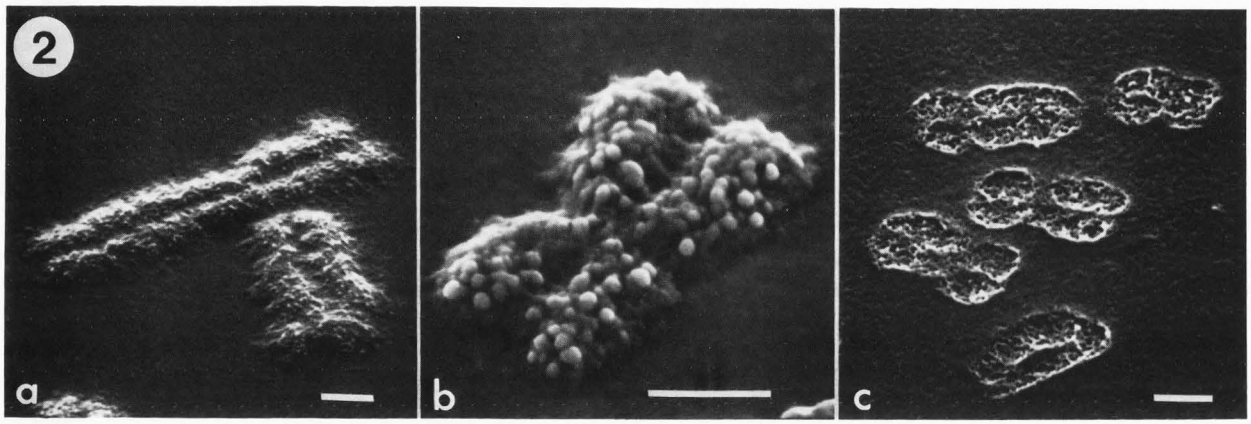


Fig. 1. Raji chromosomes uncoiled by a treatment of 1/16 isotonic solution of sodium citrate. Well uncoiled chromosomes show a coiled-coil structure (b and c). Bar = 5 μm.

Fig. 2. SEM examinations of chromosomes prepared for light microscopy. Giemsa-stained chromosomes are usually too flat to examine fine structure (a). Prolonged duration of sputter-coating (6 min) caused an artifact: a decoration effect (b). (c) represents "footprints" of chromosome. Chromosomes must have been sloughed off leaving engravings. SEM S-450. Bar = 1 μm.

a piece of double-sided adhesive tape. The specimens were sputter-coated with gold in an ion-coater (Echlin, 1975; Daskal et al., 1976). In order to achieve in SEM observation of chromosomes, well condensed and darkly stained chromosomes had to be selected during previous examination by LM.

The topology of air-dried chromosomes was variable. Some chromosomes were too flat to observe, others were granular like an ear of corn or a bunch of grapes (Figs. 2a, b). The granular appearance of the chromosomes shown in these photographs does not denote the true structure of chromosomes, but must have been derived from the combined effect of two kinds of artifact; a spike brightness effect (Holloway and Baker, 1974) and a decoration effect (Holland, 1976). Although these granules were the result of artifacts, the arrays of granules corresponded well to the spiral structure of the chromosomes observed by light microscopy.

G-banded chromosomes, C-banded chromosomes, and differentially stained sister chromatids were also examined by SEM. A common feature of topology of these chromosomes was bulkiness and rough appearance of chromosomes where the Giemsa-staining was positive. The Giemsa-positive bands seen under LM corresponded to ridges in the chromosomes observed with SEM. The height of ridges seemed to be proportional to the intensity of the Giemsa-stain. The distinctness of the ridges in the G-banded chromosomes varied with the degree of trypsinization. In C-banded chromosomes, the

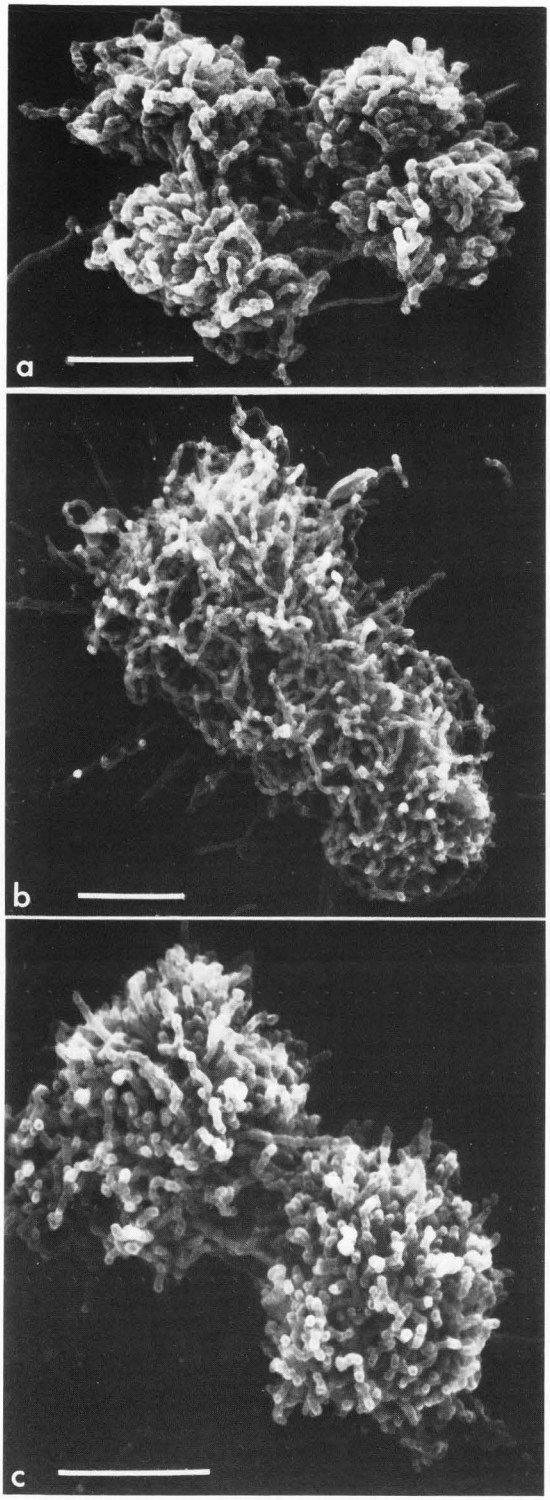


Fig. 3. Surface-spread chromosomes of Raji cells (a), mouse bone marrow cells (b), and Chinese hamster cells (c). It is a common feature among these chromosomes that they are composed of nodular and twisted looping fibers. a:FESEM S-700, b and c:FESEM S-800. Bar = 0.5 μm.

Giemsa-positive regions also appeared to be raised. The same was true in sister chromatid differential staining. The unifilarly bromodeoxyuridine-substituted chromatids which stain darkly with Giemsa were thick and their surfaces rough, whereas the bifilarly substituted chromatids which stain palely were thin and smooth (Utsumi, 1982).

The ridge found in the Giemsa-positive regions of G-banded chromosomes and the raised spot in the centromere region in C-banded chromosomes also have been reported by Ross and Gormley (1973) and Ruzicka (1974) in their studies of chromosomes by transmission replica method. In sister chromatids differentially stained by treatment with hot disodium phosphate solution, the Giemsa-positive chromatids also had a "pile up" appearance in SEM topology (Takayama et al., 1981). Clearly, Giemsa staining is responsible for the bulkiness of chromosomes. The molecules of magenta compound formed during Giemsa staining (Sumner and Evans, 1973) has been suggested to be large enough to increase the volume of the stained chromosomes, or the binding of the dye to DNA to alter the basic structure of the chromosome.

One of the most interesting findings that would not be recognized without the SEM examination was the "footprint" of chromosomes as shown in Fig. 2c (Utsumi, 1982). We often observe "transparent" chromosomes in the studies of LM preparations but disregard them as a result of the failure of staining and so forth. Transparent chromosomes have been reported by several investigations in different terms: chromosome ghost (Burkholder, 1971; Harrison et al., 1983), empty aspect (Drets et al., 1978), or bas-relief aspect (Drets and Novello, 1980). Drets and Novello (1980) even made a karyotype of the contour chromosomes obtained by their delineation method. This phenomenon seems to occur in chromosomes prepared by several different procedures: i) trypsin-treatment (Burkholder, 1971; Comings et al., 1973; Harrison et al., 1983), ii) the ASG technique (Gormley and Ross, 1972), iii) hot phosphate buffer (Barnett et al., 1973), iv) the C-banding technique (Pathak and Arrighi, 1973), v) the G-banding technique (Ross and Gormley, 1973), vi) prolonged periods of photo-oxidation (Drets et al., 1978), and vii) by potassium permanganate-sodium bisulfite (Drets and Novello, 1980). These figures may not represent the chromosomes themselves, but a kind of engraving on the cellular material spread over the substratum to which the chromosomes had been anchoring. The chromosomes must have been sloughed off leaving their "footprints" during staining procedure.

Chromosome preparations for LM study made by the ordinary air-drying method are not suited for the study of higher-order structure of chromosomes. Methanol-acetic acid (3:1), which is the common fixative in the air-drying method, might be responsible for the defectiveness. Biochemical studies on the effects of fixation in cytological preparations have shown that methanol-acetic acid (Sumner et al., 1973) as well as ethanol-acetic acid (Dick and Johns, 1967) extract histones from the nuclei and chromosomes. Of the five types of histones, H1 is the most susceptible to extraction by the fixative (Burkholder and Duczek, 1980).

Since H1 histone stabilizes the super-structure of the nucleosome (Finch and Klug, 1976), the depletion of histones caused by the use of fixative may result in the loosening of the structure of chromosomes. A loosened chromosomes easily could be crushed on the slide by the surface tension of the fixative during air-drying.

SEM Studies of Surface-Spread Chromosomes

The surface-spreading technique had been used for the electron microscopical study of nucleic acid molecules, DNA expelled from phage and bacteria, or purified DNA (Kleinschmidt, 1968). Gall (1963) first demonstrated nuclear fibers of newt erythrocytes by this technique. Then DuPraw (1965) and Wolfe (1965a) employed this technique for the preparation of chromosomes. Thus, it became possible to examine a whole chromosome in TEM. The whole mount chromosomes can be examined without fixation, staining, and coating (DuPraw, 1965), though they had been fixed with uranyl acetate and shadowed with platinum-palladium in early investigations (Gall, 1963, 1966; Wolfe, 1965a, 1965b).

Golomb and Bahr (1971) first observed surface-spread chromosomes with SEM and reported that the chromosomes consisted of tortuous looping fibers and revealed an appearance like a "skein of yarn". Utsumi (1981, 1982) confirmed their results that chromosomes were composed of nodular and twisted fibers (Fig. 3). Their overall diameters appear to be constant. Looping of the fiber is evident all along the chromosomes. Free ends of the fibers are rarely seen in minimally stretched chromosomes. No regular arrangement of the looping fibers, which might suggest a higher-order structure of chromosomes was found. Although the arrangement of looping fibers is disordered, the split between the chromatids, the kinetochore region, and gaps are discernible. In the kinetochore region, neither the particular structure reported by Ris and Witt (1981) nor the dense clumping of fibers reported by Wolfe (1965a) was found, but bundles of chromosome fibers arranged parallel to the axis of the chromatid were present. In the stretched chromosomes, the mass of fibers must have elongated, resulting in bundles of longitudinal fibers with segmental accumulation of fibers.

The twisted loops with nodules of chromosome fibers might be a remnant of the original conformation of fibers: each loop originally might have been a close-packed coil. When chromosomes were spread on the water surface, the close-packed coil had been uncoiled. Then the uncoiled fibers had been aggregated differently from the original configuration forming "extended coils" or "loops" due to dehydration with ethanol and/or desiccation by critical point-drying. The nodular structure of the fiber, therefore, might have resulted from the twist of each turn of the close-packed coil.

The "skein of yarn" (Golomb and Bahr, 1971) is an apt description for a topological feature of surface-spread chromosomes. The feature has been variously called: bumpy, lumpy, or knotty in terms of swellings and constrictions along the length of the fibers, tortuous, contorted, or kinky in terms of form, and loop for the continuous fibers emerging from the chromosome body and returning to it (Abuelo and Moore, 1969; Bahr and Engler, 1980; Gall, 1966; Golomb and Bahr, 1971, 1974; Lampert and Lampert, 1970; Wolfe, 1965a). Therefore, this topological feature of fibers is a universal feature of chromosome structure produced by the surface-spreading technique.

Similar looping fibers also have been observed in thin sections of isolated chromosomes (Adolph, 1980; Marsden and Laemmli, 1979). A "microconvule" is another topological feature of chromosomes which were isolated and fixed with uranyl acetate (Daskal et al., 1976). This feature may present the coagulated form of chromosome fibers caused by fixation.

The diameter of the chromosome fiber is consistent, between 200 and 300 Å, as reported for various techniques including examinations of thin-sectioned chromosomes by TEM (Adolph, 1980; Lampert and Lampert, 1970; Lampert, 1971), of surface-spread chromosomes by TEM (Abuelo and Moore, 1969; DuPraw and Bahr, 1969; Gall, 1966; Lampert and Lampert, 1970; Lampert, 1971; Wolfe, 1965a), and of negatively stained chromosomes by Miller's method (Rattner and Hamkalo, 1978a, b). Golomb and Bahr (1971) reported 400 Å fibers in unshadowed chromosomes observed by SEM. Utsumi also reported the diameters of chromosome fibers in his SEM studies on surface-spread chromosomes that the mean diameter of chromosome fibers of Raji cells measured at a hundred or more different sites on enlarged photographs were 336 $\pm$ 42 Å (Utsumi, 1981), 302 $\pm$ 38 Å and 298 $\pm$ 40 Å (Utsumi, 1982). That of mouse bone marrow cells and of Chinese hamster cells was 276 $\pm$ 35 Å and 326 $\pm$ 40 Å, respectively (Fig. 3). The difference in the mean diameters of these chromosome fibers was not statistically significant. The thickness of the coating should not be disregarded when measuring the net diameter of the chromosome fibers. The thickness of the coating used in this study was 33.6 Å, calculated from Echlin's equation (Echlin, 1975).

SEM Studies of Surface-Spread Nuclei

The ultrastructure of interphase nuclei has been studied in thin sections using TEM by many investigators. Electron micrographs of thin sections of interphase nuclei generally show heterogeneously condensed chromatin, a peripheral layer of condensed chromatin closely associated with the inner layers of nuclear membrane, and nucleoli surrounded by dense chromatin. The fibrous structure of chromatin is not discernible in thin sections, although it is the major component of the nucleus. The surface-spreading technique, however, provides an advantage that whole nuclei are observed. Gall (1963) first reported in his TEM study that the nuclei of the newt erythrocytes consists of fibers about 400-600 Å (subsequently revised to 250-300 Å, Gall, 1966) in diameter by the surface-spreading technique. Wolfe (1965b) also reported the fibers 250 $\pm$ 20 Å in average diameter in the nucleated erythrocytes from salamander, and DuPraw (1965) fibers 230-250 Å in diameter in nuclei of honeybee embryonic cells, in TEM studies. Golomb and Bahr (1974) and Golomb and Reese (1974) used SEM to observe chromatin fibers with uniform diameter in the surface-spread nuclei of human peripheral lymphocytes.

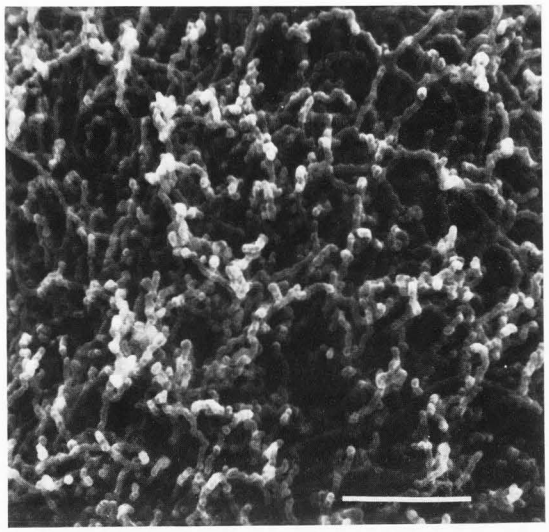


Fig. 4. Enlarged surface topology of a surface-spread nucleus of the mouse bone marrow cells. The feature of nuclear fibers is very similar to that of chromosome fibers shown in Fig. 3b. FESEM S-800. Bar = 0.5 μm.

A very few nuclei have retained their spherical form after surface-spreading. When Raji cells were spread, the majority of nuclei formed frayed disks of networks of fiber and some were completely disrupted. The extent of spreading of nuclei seemed to depend on the compactness of nuclear structure, Mouse bone marrow and splenic cells and Chinese hamster cells gave better results in retaining the spherical structure. The procedure of preparation of surface-spread nuclei is the same as that of surface-spread chromosomes (see Notes on Techniques). Spherical nuclei were selected and marked under a phase contrast microscope immediately after the critical point-drying. As shown in Fig. 4, nuclei of bone marrow cells are composed of fibers very similar in appearance to chromosome fibers: nodular, twisted looping fibers, very uniform in diameter and lack of free ends. The mean diameter of the nuclear fibers of mouse bone marrow cells obtained from a hundred measurements was 302 $\pm$ 40 Å (Fig. 4), whereas that of the chromosome fibers of the same cells on the same specimen was 276 $\pm$ 35 Å (Fig. 3b). The difference between these values was statistically significant. However, the nuclear fibers must essentially be the same as the chromosome fibers. The difference between them may be in the degree of condensation or the manner of coiling. Since the nuclear fibers are not so closely packed as those in chromosomes, the assignment of fibers to individual chromosomes is difficult. DuPraw (1966) reported in the study of human lymphocytes that the diameters are around 230 Å at interphase and have a somewhat greater dimension (possibly exceeding 300 Å) at metaphase. The discrepancy of the average fiber diameter between nuclei and chromosomes was probably due to the effect of stretching resulting from the surface-spreading technique.

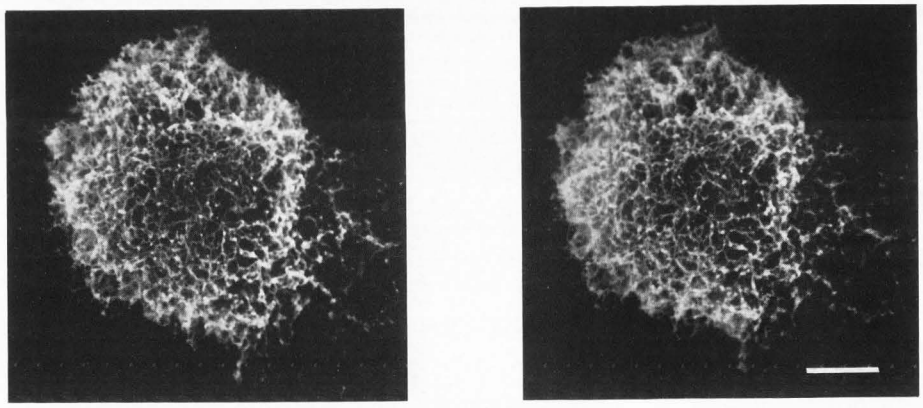


Fig. 5. Stereo-pair showing a surface-spread nucleus of the mouse bone marrow cells. SEM S-450. Bar = 1 μm.

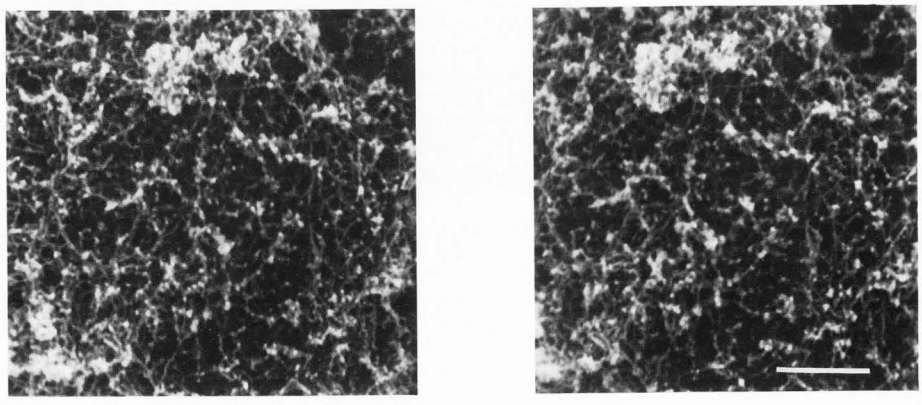


Fig. 6. Stereo-pair showing enlarged surface topology of a surface-spread nucleus of the mouse bone marrow cells. SEM S-450. Bar = 1 μm.

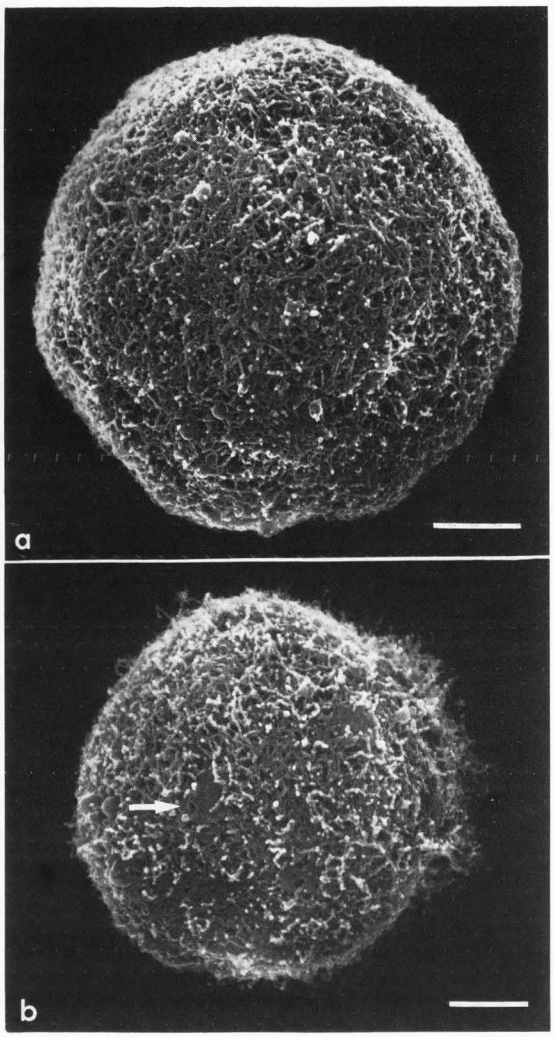


Fig. 7. Surface-spread nuclei of the mouse bone marrow cells. Nuclear fibers are rather tightly packed (a). A nuclear pore-like structure is seen in the smooth surface area which is probably the remnant of the nuclear envelope (arrow in b). SEM S-450. Bar = 1 μm.

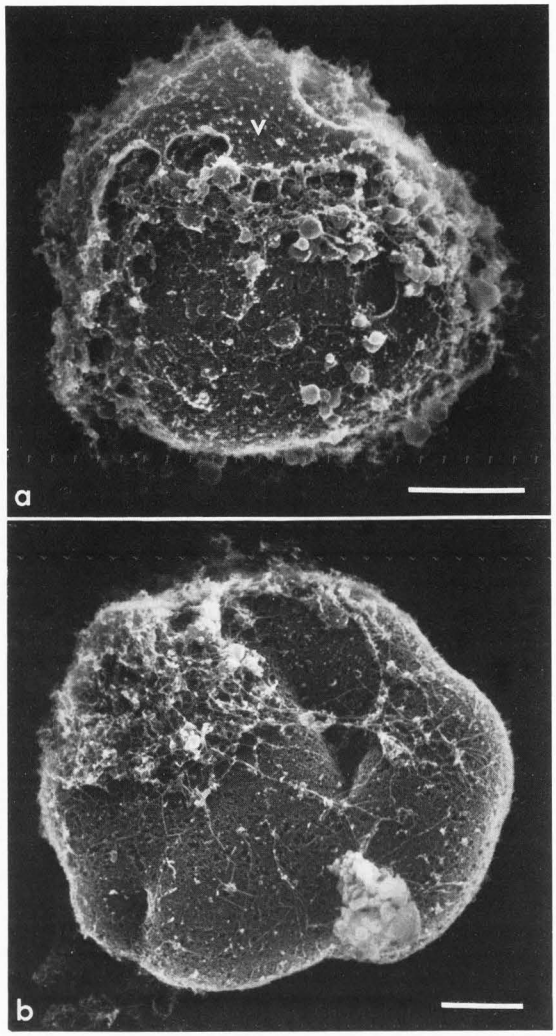


Fig. 8. Surface-spread nuclei of the Chinese hamster cells. a: An incompletely ruptured cell showing the remnant of cell membrane with villi (v). Spherules are probably swollen mitochondria. b: Cytoskeletal fibers are seen on the nucleus. SEM S-450. Bar = 1 μm.

Figures 5 and 6 show extremely unpacked nuclei in stereographs.

In some nuclei, the nuclear fibers do not loop out, but are rather tightly packed (Fig. 7a). The nuclear pores are rarely seen (Fig. 7b). Incompletely disrupted cells (Figs. 8a, b) show the remnants of cell membrane, cytoskeletal fibers, and mitochondria.

In order to study the internal structure of nuclei, the frozen resin cracking method (Tanaka and Iino, 1973) or cryofracturing method (Maruyama, 1983) may be applicable, since the distortion of nuclear structure is minimized by these methods.

Notes on Techniques

Cell Lines

Raji cells, a human tissue culture cell line derived from Burkitt lymphoma (Pulvertaft, 1964; Epstein et al., 1966), grown in RPMI-1640 supplemented with 20% fetal calf serum, and Chinese hamster ovary (CHO-K1) cells grown in MEM supplemented with 10% fetal calf serum were used.

Bone Marrow Cells

Bone marrow cells were obtained from femoral bones and tibial bones of CBA/H-T6 mice (Animals were maintained under the guidelines set forth by the Aichi Cancer Center Research Institute, Nagoya, Japan). One month-old mice received an intraperitoneal injection of colchicine, 2 μg per gram of body weight, four hours before sacrifice by cervical dislocation. Bone marrows were flushed out through the long bone with isotonic solution of sodium citrate (3.02%) by 1 ml disposable syringe and 26G needle. Bone marrow cells were suspended in 5 ml of isotonic solution of sodium citrate and centrifuged at 1000 rpm for 10 min. Sedimented cells were resuspended in a small amount of 1.0% sodium citrate solution for swelling the cells.

The resulting cell suspension was applied on the surface of the water as described below.

Surface-Spreading Technique

A rectangular trough, 50 x 70 mm and 10 mm deep, was made of a 2 mm acryl plate. A bar made of the same material, 12 x 80 mm, was used for cleaning the surface of the water. Petri dish, rectangular trough made of glass, polyvinyl chloride, Teflon, or Teflon-coated metal may be used as a "Langmuir-trough" (Kleinschmidt, 1968; Golomb, 1976). The size and shape of the trough may be arbitrary, but the rim of the trough should be ground flat and hydrophobic so as to obtain the convex surface of the water when it was filled to the brim and swept effectively using the cleaning bar. Raji cells were sedimented at 1000 rpm for 10 min, then they were resuspended in a small amount of Hanks' buffered saline solution. A tiny amount of the cell suspension (1 µl or less) was placed on the tip of a microspatula with a capillary pipet. The droplet then was placed on the convex surface of the water in the trough filled to the brim with distilled-deionized water. Just before application of the cells, a few talcum particles were dusted on the surface of the water to check the extent of spreading (for details see Gall, 1966; Kleinschmidt, 1968; Golomb, 1976). The surface tension of the water ruptured the cells, releasing nuclei and chromosomes which were picked up by touching a piece of Formvar-coated glass to the surface. The pieces of glass were made of microscope slides cut into four equal parts and coated with Formvar (0.5% in chloroform). The specimens were dehydrated, for approximately three minutes each, with ascending concentrations of ethanol (30, 50, 70, 90%, and two changes of absolute ethanol). The specimens then were put through three changes of isoamyl acetate, after which they were critical point-dried from liquid carbon dioxide (Anderson, 1951). The specimens may be stored in 90% ethanol until used. The volume fraction of isoamyl acetate permissible in liquid carbon dioxide is very small (0.023%) as compared with other intermediate fluids such as acetone, ethanol, and Freon 113 (Pawley and Dole, 1976). Therefore, isoamyl acetate taken with the specimen into the critical point-drier should be as meager as possible. An excess amount of isoamyl acetate may cause incomplete dryness and influence the final figure of chromosomes. Isoamyl acetate wasremoved from the specimen with a piece of blotting paper taking care that the specimen did not dry up.

Subphase

To study the higher-order structure of chromosomes, undistorted chromosomes are preferable. The stretching of chromosomes, however, is inevitable so far as the surface-spreading technique is used. Attempts were made to reduce the surface tension of the water by mixing with several different detergents or ethanol. But chromosomes were disturbed by the solute itself, too. Finally, it was found that the extent of the stretching of chromosomes was somewhat smaller on deuterium oxide than on the water, probably because the surface tension of the former (67.8 dyne/cm at 20°C) is smaller than the latter (72.75 dyne/cm at 20°C). Deuterium oxide was used as hypophase to stabilize microtubules (Ris and Witt, 1981).

Sputter-Coating

The specimens were sputter-coated with gold in an ion-coater at 1400 V. The electric current was kept at 8 mA by leaking the air. Sputtering for 30 seconds gave a minimum thickness of coating that was enough to protect from charging. The thickness of the gold coating is 33.6 Å, according to Echlin's equation (Echlin, 1975). The more the duration of sputtering was prolonged, the coating became thicker and caused artifacts such as the decoration effect (Holland, 1976) and the edge contrast or spike brightness effect (Holloway and Baker, 1974).

Scanning Electron Microscopy

The specimens were examined with a Hitachi SEM S-450 and/or a Hitachi FESEM S-700 and S-800. Photographs were made on Polaroid type 665 P/N film or Kodak Tri-X Pan film.

Stereography

For stereography, photographs taken at a tilt angle of 15° were placed on the left side of the pairs, and photographs taken at 25° were placed on the right.

Conclusions

By the surface-spreading technique, metaphase chromosomes or interphase nuclei proved to be composed of fibers about 300 Å in diameter. The diameter of fibers is consistent and free ends of the fibers are rarely seen. The fibers are nodular, twisted and looping. The chromosome fibers are not distinguishable from the nuclear fibers by their appearance. The value for the diameter of these fibers well coincide with that of the solenoidal model for chromatin proposed by Finch and Klug (1976). These values also coincide with the value obtained by Rattner and Hamkalo (1978a, b) and Rattner et al. (1981) in the study of negatively stained chromosomes prepared by Miller's method (Miller and Bakken, 1972). They reported that the 200-300 Å fibers were composed of closely apposed arrays of nucleosomes.

The total length of the DNA double helix in human cells is 190 cm calculated from the number of nucleotide pairs (5.6×10^9, McCarthy, 1969) and the base distance in the DNA molecule (3.4 Å, Watson and Crick, 1953). Since the total length of the human metaphase chromosomes is about 220 µm (the average value obtained from five karyotypes), the DNA double helix has to be packed in a ratio of about 8600:1 to form the metaphase chromosomes. The first supercoiling of the DNA double helix is found in cucleosomes in which the repeating unit contains 200 base pairs at a distance of 125 Å (Olins and Olins, 1974; Kornberg, 1974; Oudet et al., 1975). Thus the DNA double helix is packed in a ratio of 5:1 to form nucleosomes. Since the solenoid is a supercoil of closely-packed nucleosomes with six to seven nucleosomes per turn (Finch and Klug, 1976), the DNA double helix is packed in a ratio of 30-35:1 to form the solenoid. Therefore, the chromosome fibers revealed by the SEM study must further be packed in a ratio of 250-290:1 to form the metaphase chromosomes. In other words, if one could unravel the mass of chromosome fibers shown in the photographs, there would be a single fiber 250-290 times as long as the chromatid.

The kinetochore region was seen as constrictions or gaps between the masses of tortuous chromosome fibers. In the stretched chromosomes, a bundle of several chromosome fibers was seen at the kinetochore region. However, the attachment of microtubules to chromosome fibers, which was observed in the kinetochore region by Ris and Witt (1981) in their TEM study of whole mount chromosomes, was not recognized. There was no structure suggesting the "unit fiber" described by Bak and Zeuthen (1978), in either stretched or unstretched chromosomes.

Chromosomes are generally prone to stretch lengthwise, when they were spread on the water (Fig. 9). Chromomeres, or segmental masses of chromosome fibers are seen in moderately stretched chromosomes (Fig. 10a). In the extremely stretched chromosomes, the chromosome fibers are no longer tortuous or looped, but show a bundle of straight fibers. Broken ends of the chromosome fibers are often seen in such an extremely stretched chromosome (Fig. 10b). Based on observations of whole mount chromosomes at various stages of stretching, it is concluded that there is no other type of fiber which is suggestive of the scaffold structure (Paulson and Laemmli, 1977) than 300 Å fibers. The scaffold structure was denied by Okada and Comings (1980) that it was an artifact resulting from incomplete dispersion of central chromatin and aggregation of nonhistone proteins in dehistonized chromosomes.

Although it has been clearly shown in many studies that the 200-300 Å fibers are the structural element of the chromosome, the manner of organization by which these fibers build up the higher-order structure of the chromosomes is still a point of discussion. DuPraw (1966) postulated a "folded-fiber" model and Bahr (1970) a "coiled-coil" model for metaphase chromosomal structure based on their observations of surface-spread chromosomes by TEM. Marsden and Laemmli (1979), however, put forth a "radial loop" model based on electron micrographs of thin sections of isolated chromosomes. The cross-sections of isolated chromosomes suggest the axial structure of the chromosomes (Adolph, 1980; Marsden and Laemmli, 1979). But in chromosomes severely stretched by the surface-spreading technique, neither core nor axial elements are recognizable (Bahr, 1977; Utsumi, 1981, 1982). The differences in the presence or absence of the axial structure of the chromosome may be due to the different techniques for chromosome preparation.

Acknowledgements

I am indebted to Dr. Yoshimi Nishi, Researcher of the Laboratory of Ultrastructure Research of this Institute, for his valuable advice on the operation of the scanning electron microscope, S-450. Thanks also are due to Naka Works, Hitachi Ltd., for the generous offering in the use of the field emission type scanning electron microscopes, FESEM S-700 and S-800, and to Mr. Mitsuhiko Yamada of the Application Laboratory, Naka works, Hitachi Ltd., for his expert cooperation.

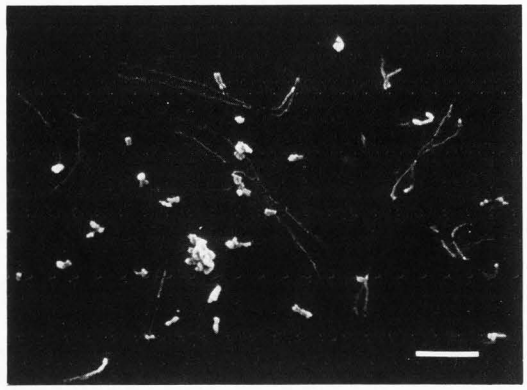


Fig. 9. A metaphase plate of Raji cells surface-spread on the water. Chromosomes are prone to stretch. Minimally stretched chromosomes are suited to the study of the higher-order structure. SEM S-450. Bar = 10 μm.

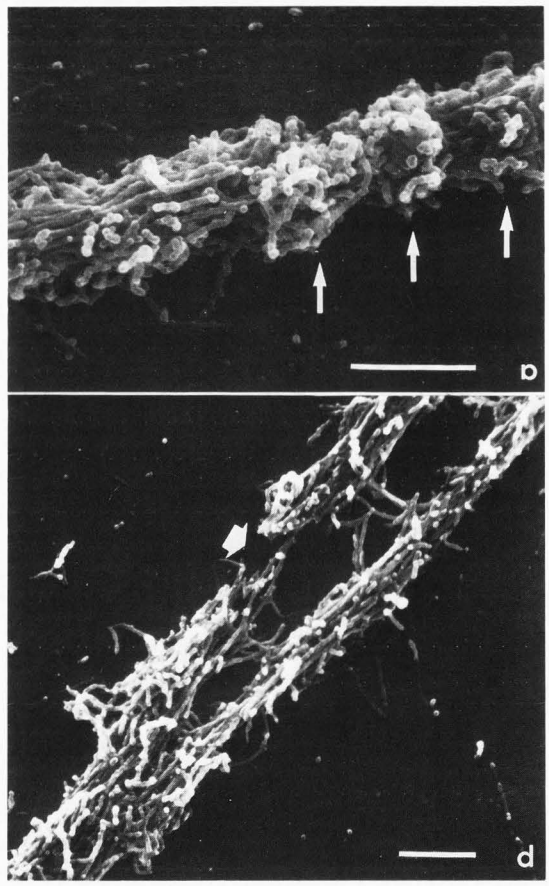


Fig. 10. Surface-spread chromosomes of Raji cells. A part of stretched chromosome showing segmental masses of chromosome fibers or chromomeres (arrows in a). The masses of fibers are no longer seen in the extremely stretched chromosome (b). The fibers are often torn in such a stretched chromosome (arrow). FESEM S-700 (a) and S-800 (b). Bar = 0.5 μm.

References

Abuelo JG, Moore DE (1969) The human chromosome. Electron microscopic observations on chromatin fiber organization. J. Cell Biol., 41, 73-90.

Adolph KW (1980) Isolation and structural organization of human mitotic chromosomes. Chromosoma (Berl.), 76, 23-33.

Anderson TF (1951) Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. Trans. N. Y. Acad. Sci., Ser. II, 13, 130-134.

Angelier N, Paintrand M, Lavaud A, Lechaire JP (1984) Scanning electron microscopy of amphibian lampbrush chromosomes. Chromosoma (Berl.), 89, 243-253.

Bahr GF (1970) Human chromosome fibers. Considerations of DNA-protein packing and of looping patterns. Exp. Cell Res., 62, 39-49.

Bahr GF (1977) Chromosomes and chromatin structure, in: Molecular Structure of Human Chromosomes, Yunis JJ (ed.), Academic Press, New York, 143-203.

Bahr GF, Engler WF (1980) Artifacts observed in critical point-dried preparations of human chromosomes by electron microscopy. J. Ultrastr. Res., 73, 27-33.

Bak AL, Zeuthen J (1978) Higher-order structure of mitotic chromosomes. Cold Spr. Harb. Symp. Quant. Biol., 42, 367-377.

Baranetzky J (1880) Die Kernteilung in den Pollenmutterzellen einiger Tradescantien. Bot. Zeitg., 38, 241.

Barnett RI, MacKinnon EA, Romero-Sierra C (1973) Delineation of human chromosome contour by heat treatment and hematoxylin staining. Chromosoma (Berl.), 40, 299-306.

Burkholder GD (1971) Electron microscopic visualization of chromosomes banded with trypsin. Nature (Lond.), 247, 292-294.

Burkholder GD (1981) The ultrastructure of R-banded chromosomes. Chromosoma (Berl.), 83, 473-480.

Burkholder GD, Duczek LL (1980) Proteins in chromosome banding I. Effect of G-banding treatments on the proteins of isolated nuclei. Chromosoma (Berl.), 79, 29-41.

Christenhuss R, Büchner Th, Pfeiffer RA (1967) Visualization of human somatic chromosomes by scanning electron microscopy. Nature (Lond.), 216, 379-380.

Comings DE, Avelino E, Okada TA, Wyandt HE (1973) The mechanism of C- and G-banding of chromosomes. Exp. Cell Res., 77, 469-493.

Daskal Y, Mace Jr ML, Wray W, Busch H (1976) Use of direct current sputtering for improved visualization of chromosome topology by scanning electron microscopy. Exp. Cell Res., 100, 204-212.

Dick C, Johns EW (1967) The removal of histones from calf thymus deoxyribonucleoprotein and calf thymus tissue with acetic acid containing fixative. Biochem. J., 105, 46p.

Drets ME, Folle GA, Comings DE (1978) Mechanism of chromosome banding X. Chromosome and nuclear changes induced by photo-oxidation and their relation to R-banding with anti-C antibodies. Chromosoma (Berl.), 69, 101-112.

Drets ME, Novello A (1980) Chromosome delineation induced by a combined potassium permanganate-sodium bisulfite treatment. Chromosoma (Berl.), 78, 371-376.

DuPraw EJ (1965) The organization of nuclei and chromosomes in honeybee embryonic cells. Proc. Natl. Acad. Sci. USA, 53, 161-168.

DuPraw EJ (1966) Evidence for a 'folded-fiber' organization in human chromosomes. Nature (Lond.), 209, 577-581.

DuPraw EJ, Bahr GF (1969) The arrangement of DNA in human chromosomes, as investigated by quantitative electron microscopy. Acta Cytologica, 13, 188-205.

Echlin P (1975) Sputter-coating techniques for scanning electron microscopy. Scanning Electron Microsc. 1975: 217-224.

Epstein MA, Achong BG, Barr YM, Zajac B, Henle G, Henle W (1966) Morphological and virological investigations on cultured Burkitt tumor lymphoblasts (Strain Raji). J. Natl. Cancer Inst., 37, 547-559.

Finch JT, Klug A (1976) Solenoidal model for superstructure in chromatin. Proc. Natl. Acad. Sci. (Wash.), 73, 1897-1901.

Gall JG (1963) Chromosome fibers from an interphase nucleus. Science, 139, 120-121.

Gall JG (1966) Chromosome fibers studied by a spreading technique. Chromosoma (Berl.), 20, 221-233.

Golomb HM (1976) Human chromatin and chromosomes studied by scanning electron microscopy: Progress and prospectives. J. Reprod. Med., 17, 29-35.

Golomb HM, Bahr GF (1971) Scanning electron microscopic observations of surface structure of isolated human chromosomes. Science, 171, 1024-1026.

Golomb HM, Bahr GF (1974) Human chromatin from interphase to metaphase. A scanning electron microscopic study. Exp. Cell Res., 84, 79-87.

Golomb HM, Reese C (1974) Human interphase chromatin: A three-dimensional presentation. Lancet, April 27, 806-807.

Gormley IP, Ross A (1972) Surface topology of human chromosomes examined at each stage during ASG banding procedure. Exp. Cell Res., 74, 585-587.

Harrison CJ, Britch M, Allen TD, Harris R (1981) Scanning electron microscopy of the G-banded human karyotype. Exp. Cell Res., 134, 141-153.

Harrison CJ, Allen TD, Britch M, Harris R (1982) High-resolution scanning electron microscopy of human metaphase chromosomes. J. Cell Sci., 56, 409-422.

Harrison CJ, Allen TD, Harris R (1983) Scanning electron microscopy of variations in human metaphase chromosome structure revealed by Giemsa banding. Cytogenet. Cell Genet., 35, 21-27.

Holland VF (1976) Some artifacts associated with sputter-coated samples observed at high magnification in the scanning electron microscope. Scanning Electron Microsc. 1976; I: 71-74.

Holloway PJ, Baker EA (1974) The aerial surface of higher plants, in: Principles and Techniques of Scanning Electron Microscopy, Hayat MA (ed.), Van Nostrand Reinhold, New York, Vol. 1, 181-205.

Kleinschmidt AK (1968) Monolayer techniques in electron microscopy of nucleic acid molecules, in: Methods in Enzymology, Grossman L, Moldave K (eds.), Academic Press, New York, Vol. 12B, 361-377.

Kornberg RD (1974) Chromatin structure: A repeating unit of histones and DNA. Science, 184, 868-871.

Kuwada Y (1939) Chromosome structure, A critical review. Cytologia, 10, 213-256.

Laane MM, Wahlstrøm R, Mellem TR (1977) Scanning electron microscopy of nuclear division stages in Vicia faba and Haemanthus cinnabarinus. Hereditas, 86, 171-178.

Lampert F (1971) Coiled supercoiled DNA in critical point dried and thin sectioned human chromosome fibers. Nature (Lond.) New Biol., 234, 187-188.

Lampert F, Lampert P (1970) Ultrastructure of human chromosome fiber. Humangenetik, 11, 9-17.

Marsden MPF, Laemmli UK (1979) Metaphase chromosome structure: Evidence for a radial loop model. Cell, 17, 849-858.

Maruyama K (1983) Stereoscopic scanning electron microscopy of the chromosomes in Vicia faba (broad beans). J. Ultrastr. Res., 82, 322-326.

McCarthy BJ (1969) The evolution of base sequences in nucleic acids, in: Handbook of Molecular Cytology, Lima-de-Faria A (ed.), North-Holland, Amsterdam, 3-20.

Meyer GF, Lipps HJ (1984) Electron microscopy of surface spread polytene chromosomes of Drosophila and Stylonichia. Chromosoma (Berl.), 89, 107-110.

Miller Jr OL, Bakken AH (1972) Morphological studies of transcription. Acta endocr. (Kbh.), 168, 155-177.

Mullinger AM, Johnson RT (1983) Units of chromosome replication and packing. J. Cell Sci., 64, 179-193.

Nakanishi YH, Utsumi S, Liu I-M (1970) Demonstration of spiral structure of chromosomes by high voltage electron microscopy. Jpn. J. Genet., 45, 313-315.

Neurath PW, Ampola MG, Vetter HG (1967) Scanning electron microscopy of chromosomes. Lancet II, 1366-1367.

Ohnuki Y (1968) Structure of chromosomes I. Morphological studies of the spiral structure of a human somatic chromosomes. Chromosoma (Berl.), 25, 402-428.

Okada TA, Comings DE (1980) A search for protein cores in chromosomes: Is the scaffold an artifact? Am. J. Hum. Genet., 32, 814-832.

Olins AL, Olins DE (1974) Spheroid chromatin units (ν bodies). Science, 183, 330-332.

Oudet P, Gross-Bellard M, Chambon P (1975) Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. Cell, 4, 281-300.

Pathak S, Arrighi FE (1973) Loss of DNA following C-banding procedures. Cytogenet. Cell Genet., 12, 414-422.

Paulson JR, Laemmli UK (1977) The structure of histone depleted metaphase chromosomes. Cell, 12, 817-828.

Pawley JB, Dole S (1976) A totally automatic critical point dryer. Scanning Electron Microsc. 1976; I: 287-294.

Pawlowitzki IH, Blaschke R (1971) Preparing chromosomes for scanning electron microscopy without enzyme digestion. J. Microsc., 93, 119-122.

Pawlowitzki IH, Blaschke R, Christenhuss R (1968) Darstellung von Chromosomen im Raster-Elektronenmikroskop nach Enzymbehandlung. Naturwiss., 55, 63-64.

Pulvertaft RJV (1964) Cytology of Burkitt's tumor (African lymphoma). Lancet 1, 238-240.

Rattner JB, Hamkalo BA (1978a) Higher order structure in metaphase chromosomes I. The 250 Å fiber. Chromosoma (Berl.), 69, 363-372.

Rattner JB, Hamkalo BA (1978b) Higher order structure in metaphase chromosomes II. The relationship between the 250 Å fiber, superbeads and beads-on-a-string. Chromosoma (Berl.), 69, 373-379.

Rattner JB, Goldsmith MR, Hamkalo BA (1981) Chromosome organization during male meiosis in Bombyx mori. Chromosoma (Berl.), 82, 341-351.

Ris H, Witt PL (1981) Structure of mammalian kinetochore. Chromosoma (Berl.), 82, 153-170.

Ross A, Gormley IP (1973) Examination of surface topology of Giemsa-banded human chromosomes by light and electron microscopic techniques. Exp. Cell Res., 81, 79-86.

Ruzicka F (1974) Organization of human mitotic chromosomes. Humangenetik, 23, 1-22.

Stubblefield E, Wray W (1971) Architecture of the Chinese hamster metaphase chromosomes. Chromosoma (Berl.), 32, 262-294.

Sumner AT, Evans HJ (1973) Mechanisms involved in the banding of chromosomes with quinacrine and Giemsa II. The interaction of the dyes with the chromosomal components. Exp. Cell Res., 81, 223-236.

Sumner AT, Evans HJ, Buckland RA (1971) New technique for distinguishing between human chromosomes. Nature New Biol., 232, 31-32.

Sumner AT, Evans HJ, Buckland RA (1973) Mechanisms involved in the banding of chromosomes with quinacrine and Giemsa I. The effects of fixation in methanol-acetic acid. Exp. Cell Res., 81, 214-222.

Sweney LR, Lam LF-H, Shapiro BL (1979) Scanning electron microscopy of uncoated human metaphasic chromosomes. J. Microsc., 115, 151-160.

Takayama S, Utsumi KR, Sasaki Y (1981) Topographic examination of sister chromatid differential staining by Nomarski interference microscopy and scanning electron microscopy. Chromosoma (Berl.), 82, 113-119.

Tanaka K, Iino A (1973) Demonstration of fibrous components in hepatic interphase nuclei by high resolution scanning electron microscopy. Exp. Cell Res., 81, 40-46.

Utsumi KR (1976) The revelation of the spiral structure of chromosomes by treatment with hypotonic solution. J. Cell Biol., 70, 280a.

Utsumi KR (1981) Studies on the structure of chromosomes II. Chromosome fibers as revealed by scanning electron microscopy. Cell Struct. Funct., 6, 395-401.

Utsumi KR (1982) Scanning electron microscopy of Giemsa-stained chromosomes and surface-spread chromosomes. Chromosoma (Berl.), 86, 683-702.

Utsumi KR, Tanaka T (1975) Studies on the structure of chromosomes I. The uncoiling of chromosomes revealed by treatment with hypotonic solution. Cell Struct. Funct., 1, 93-99.

Watson JD, Crick FHC (1953) Molecular structure of nucleic acids. Nature (Lond.), 171, 737-738.

Wolfe SL (1965a) The fine structure of isolated metaphase chromosomes. Exp. Cell Res., 37, 45-53.

Wolfe SL (1965b) The fine structure of isolated chromosomes. J. Ultrastr. Res., 12, 104-112.

Xu X, Wu M (1983) Electron microscopy of G-banded human mitotic chromosomes. Chromosoma (Berl.), 88, 237-240.

Yu CK (1971) Structure of chromosomes visualized by scanning electron microscopy. Can. J. Genet. Cytol., 13, 690-696.

Discussion with Reviewers

T. D. Allen: Fig. 2b. It is difficult to assign the "knobby" appearance of chromosomes in the figure with imaging artifact as suggested in the text. Could not the swollen surface be more likely a result of change in the actual chromosome fibers during preparation?
Author: The knobby appearance is not a result of swelling of the chromosome fibers, but a deposition of gold accumulated on the protruded parts of the chromosomal surface produced by sputter-coating. Knobs are very small by 30 sec sputtering as shown in Fig. 2a. They become large after 6 min sputtering as shown in Fig. 2b.

T. D. Allen: There is an alternative explanation to chromosome 'footprints', which is that if the chromosomes are over-treated with whatever agent produces the stain banding pattern, they collapse, although without spreading out. Could this be the case for the chromosomes in Fig. 2c?
Author: The 'transparent' chromosomes can be produced under moderate conditions,e.g. 10 sec treatment with 0.025% trypsin at 23°C. Usually the ordinarily air-dried chromosomes show a fringe of fibers around them as shown in Fig. 2a. These chromosomes must have been spread directly on the glass. But 'footprints' of chromosomes show distinct contours (Fig. 2c). Chromosomes must have been spread on or partly buried in the thick layer of protein substance simultaneously spread on the glass. Since the protein substance is stained blue with Giemsa, the footprints appear transparent by LM. Adhesion of chromosomes on the protein substance must be so weak that chromosomes are readily sloughed off.

T. D. Allen: Our own investigations (Harrison et al., text references and J. Med. Genetics, 22, 16-23 (1985) and Chromosoma, 91, 363-368 (1985)) have shown consistently that the air-drying which occurs in LM metaphase spread preps from human lymphocytes can be 'overcome' by rehydration followed by glutaraldehyde and osmium-TCH fixation and impregnation. Would you please comment on this?
Author: It is one of the outstanding contributions to the study of chromosomes by SEM that Harrison and her colleagues could resuscitate the flat chromosomes made by the air-drying technique by GA-Os-TCH method. However, judging from G-banded chromosomes observed by LM in their reports, the trypsinization seems to be somewhat weak. To my disappointment, although the surface topology is suggestive of the fibrous element of chromosomes, the higher-order architecture of chromosomes can not be discerned.

T. D. Allen: The appearance of the waterspread chromosomes is one of marked dispersion of the fiber organization. Have such preparations been also observed in the LM to check the level of distortion? Furthermore, the author himself quotes stretching as a result of the water spreading technique.
Author: Surface-spread chromosomes were routinely examined by phase contrast microscopy immediately after the critical point drying. Although chromosome fibers look dispersed by SEM (Fig. 3a, b, and c), their size is a little smaller than that of chromosomes ordinarily prepared for LM. Minimally stretched chromosomes in the surface-spread metaphase (Fig. 9) look just as normal by LM.

The looping aspect of chromosome fibers may be one of distortion produced by the surface-spreading method. Coils of chromosome fibers must have been uncoiled by submerging in water which dissolved a certain substance responsible for coiling. Twisting and nodules of chromosome fibers must be the remnant of such coils. The stretching of chromosomes may be another distortion. Chromosomes are prone to stretch lengthwise rather than widthwise when they are spread on the water, though the surface tension of the water is acting on chromosomes in all directions. This denotes that the binding force between chromosome fibers in the condensed metaphase chromosomes is weaker in the direction of length than in the direction of width. Histone-depleted chromosomes, on the contrary, spread in all directions showing a halo of DNA strands around a 'scaffold' (Paulson & Laemmli, 1977). The ability of unfolding of chromosome fibers seems to be removed by histone-depletion from chromosomes.

S. M. Gollin: The use of methanol:acetic acid as a fixative for chromosomes for LM, TEM, and SEM is widespread. This fixative is the only one which permits fixation of chromosomes in situ and permits chromosome spreading. It does alter histone composition, but does not appear to drastically alter chromatin arrangement within chromosomes. In contrast, the surface spreading methods clearly alter chromatin arrangement within chromosomes, although I do not have data on what they do to chromosomal proteins. Could you please comment?
Author: Although the arrangement of chromosome fibers might have been distorted by the surface-spreading method as mentioned above and in the text, this method has the advantage of others. How can we know the higher-order structure of chromosomes which were closely packed? Internal structure of chromosomes cannot be learned from the surface topology, even if they were fixed in situ. Various degrees of unfolding of chromosomes made by the surface-spreading method may be able to provide better understanding of the higher-order structure of chromosomes.

F. Lampert: Both SEM or TEM so far have failed in establishing the method for routine cytogenetic application utilizing the higher resolution as compared to LM. An easy method is still lacking for proper identification of individual chromosomes of the human karyotype at the EM level.
Author: I wholeheartedly agree with this comment.

Editor's Note: Readers should find 3 papers on chromosomes on pages 869, 879 and 889 in Scanning Electron Microscopy/1985/II also of interest.