Scanning Electron Microscopy

Volume 1985
Number 2 *Part II*

Article 37

5-13-1985

A Scanning Electron Microscopic Technique for Three-Dimensional Visualization of the Spatial Arrangement of Metaphase, Anaphase and Telophase Chromatids

David A. Welter
Medical College of Georgia

Lon D. Hodge
Medical College of Georgia

SCANNING ELECTRON MICROSCOPY/1985/II (Pages 879-888)
SEM Inc., AMF O'Hare (Chicago), IL 60666 USA

0586-5581/85$1.00+.05

A SCANNING ELECTRON MICROSCOPIC TECHNIQUE FOR THREE-DIMENSIONAL VISUALIZATION OF THE SPATIAL ARRANGEMENT OF METAPHASE, ANAPHASE AND TELOPHASE CHROMATIDS

David A. Welter* and Lon D. Hodge

Departments of Anatomy, and Cell and Molecular Biology
Medical College of Georgia
Augusta, Georgia 30912

(Paper received January 29 1985, Completed manuscript received May 13 1985)

Abstract

Chromosome and chromatid alignment in mitotic configurations remains a topic of interest because there is little precise information. For example, reconstruction of mitotic configurations from serial sections collected with transmission electron microscopy has proven to be neither practical nor a sensitive method for conceptualizing these arrangements. Similarly light microscopy has been even more unsatisfactory because of its limited resolution and lack of three-dimensional capabilities. These limitations conceivably could be overcome by visualization of mitotic configurations by scanning electron microscopy (SEM). However, SEM has its limitations, of which the most obvious with regard to visualization of mitotic configurations, is that such structures in dividing cells are obscured from the beam by membranes, cellular organelles, and the mitotic apparatus. These "contaminants," we have found, can be removed by the appropriate procedure such that a direct three-dimensional visualization of intact life-like mitotic configurations of chromatids from mammalian cells is possible. We also demonstrate that these configurations, although some artifacts may exist, retain the same basic shape and chromatid arrangements throughout metaphase, anaphase, and telophase when compared to configurations isolated with a non-ionic detergent and neutral buffers.

Keywords: Acid Isolation Procedure, Anaphase Chromatid Orientation, Chromosome Structure, $HeLaS_3$ Cells, Interchromosomal Fibers, Metaphase Chromosomes, Mitosis, Nuclear Reformation, Scanning Electron Microscopy, Telophase Chromatid Orientation.

*Address for Correspondence:
D.A. Welter
Department of Anatomy
Medical College of Georgia
Augusta, GA 30912
Phone No. (404) 828-2022

Introduction

Developments in high resolution scanning electron microscopy (SEM) coupled with improvements in specimen preparation have enabled biologists to obtain three-dimensional perspectives of cells and tissue complexes (Harrison et al., 1981; Harrison et al., 1983; Katsumoto et al., 1981; Kalisch and Jacob, 1983; Heneen, 1981; Welter et al., 1979; Leick et al., 1979; Wray and Stubblefield, 1970; Ip and Fischman, 1979; Laane et al., 1977; Golomb and Bahr, 1971; Utsumi, 1981; Utsumi, 1982). However only cell surface and individual chromosome morphology has been studied because of the difficulty in obtaining cellular components free of obscuring membranes and cytoskeletal elements.

The application of most SEM techniques to cellular organelles, and especially to intact metaphase plates, involves technical difficulties (Golomb and Bahr, 1971; Wray and Stubblefield, 1970). Not only are metaphase chromosomes rather labile structures that are difficult to preserve in a life-like manner, but also they are surrounded by numerous mitotic organelles which obscure them from the electron beam making SEM analysis difficult (Heneen, 1981). Ideally a fixative, that would preserve chromosome arrangement without significant molecular changes and that would not cross-link cytoplasmic proteins so that they could subsequently be removed, would permit a study of life-like metaphase chromosomal arrangements.

Acid-alcohol fixation is the standard method of fixation for cytogenetic and histochemical study of metaphase chromosomes with light microscopy. The advantage of this fixation is that gross structures are preserved, and that there is limited extraction of histones and non-histone proteins (Burkholder, 1974). Numerous studies have been reported in which acid- alcohol fixed chromosomes are processed for electron microscopic analysis (Barnicott and Huxley, 1961; Neurath et al., 1967; Ford et al., 1968; Ruzicka 1971; Emmerich et al., 1973; Burkholder, 1974; Barnett et al., 1974; Vincent et al., 1975; Felluga & Martinucci, 1976; Lubit et al., 1976; Ris, 1979; Takayama et al., 1981; and Johnson et al., 1982). However, acid-alcohol fixation produces metaphase chromosomes with low contrast for SEM and limited resolution for transmission electron microscopy (TEM) and, therefore, most investigators find that acid-alcohol fixed metaphase chromosomes are unsuitable for detailed analysis of structure.

However, recently we have found (Welter, et al., 1982, 1983; Black et al., 1984) that an acid-alcohol fixation permitted SEM

Table 1. Morphometric Analysis of Mitotic Configurations from a Polar Perspective

Configuration	Isolation Procedure	Area (μm^2)	Perimeter (μm)	Diameter (μm)
Metaphase	*	57 ± 10.1	48 ± 9.3	12.4 ± 2.1
	**	55 ± 8.3	46 ± 7.1	11.5 ±3.1
	***	55 ± 7.2	46 ± 6.5	11.3 ± 2.6
Anaphase	*	29.5 ± 5.4	25.8 ± 2.8	7.2 ± 0.7
	**	26.9 ± 6.5	24.1 ± 1.7	2.8 ± 0.9
	***	27.8 ± 7.5	24.7 ± 2.2	6.8 ± 0.9
Early Telophase	*	26.1 ± 2.9	25.0 ± 4.5	7.2 ± 0.6
	**	25.7 ± 2.4	24.1 ± 2.9	6.9 ± 0.5
	***	25.6 ± 2.1	24.0 ± 3.2	6.8 ± 0.7

Measurement and analysis were made using a supplied program with an Apple II computer.

*Acetic acid method for preparation of mitotic configurations.

**Non-ionic detergent isolation procedure of Paulson for collection of mitotic configurations (Paulson, 1982).

***Neutral pH buffer isolation procedure of Wray and Stubblefield for collection of mitotic configurations (Wray and Stubblefield, 1970).

visualization of mitotic chromatid configurations during nuclear reformation in cultured mammalian cells. This procedure has, compared to other isolation methods and commonly used fixatives, distinct advantages. For example, synchronized HeLa cells collected in the absence of colcemid contained metaphase plates which remained intact, with a minimum of protein cross-linking, thus permitting further manipulation of these intact metaphase, as well as anaphase and telophase chromatid configurations.

In this study we compare, morphologically, the arrangement of metaphase, anaphase and telophase chromatids prepared following acetic acid isolation, a hexylene glycol isolation of Wray and Stubblefield (1970), and a non-ionic detergent technique of Paulson (1982). The acid isolation technique preserves the chromosomal fine structure, the arrangement and shape of the mitotic chromatid configurations and removes all membranes, cytoplasmic organelles, and mitotic components which might otherwise obscure the electron beam. Whereas the other two procedures are less than satisfactory for analysis of chromatid alignment in late mitotic cells. We also discuss the advantages and disadvantages of preparing mammalian chromatid configurations with acid-alcohol fixation for scanning electron microscopy.

Materials and Methods

$HeLaS_3$ cultures, human derived cells, were maintained in suspension culture at 37°C and were synchronized in mitosis by selective detachment after a double-thymidine blockade, as previously described (Simmons et al., 1973). Synchronized preparations had a metaphase index of at least 90% and a mitotic index greater than 96%. Incubation of the collected metaphase cells at 37°C for 25 to 40 min. provided enriched populations of anaphase and telophase cells. Chromosome number in this cell line was in the range of 62 to 68 with a mode of 65. Triploidy for each human chromosome except numbers 3, 8, 13 and 19 and four to six unidentified chromosomes were present based on Giemsa staining (Arrighi and Hsu, 1974).

LN cells (diploid human) were maintained in monolayer culture at 37°C in Hamm's F-12 medium supplemented with 20% fetal calf serum. Mitotic cells were obtained by selective detachment from cells in logarithmic growth.

Preparation of Chromatid Configurations

Metaphase chromosome plates and late mitotic chromatid configurations were prepared by detergent washes followed by sedimentation through 20% sucrose and flotation in Percoll gradients (Paulson, 1982), or in 0.05 mM $CaCl_2$ (Wray and Stubblefield, 1970), or by a modified methanol acid procedure (Ris, 1978) routinely used to prepare chromosomes for karyotype analysis. With the latter procedure, 5 to 6 $\times$ 10^6 cells synchronized in metaphase or late mitosis were washed in Earle's salts, collected by centrifugation at 600g, and then hypotonically swollen in 15.0 ml of 0.075 M KCl for 5 min. After centrifugation at 600g, the pellet was suspended in fixative composed of methanol:glacial acetic acid (3:1 v/v;) for 24 hr. Following two washes in the same fixative (3:1), structures were applied with a Pasteur pipette to clean #2–22 mm diameter cover slips and permitted to dry for 5 sec. The cells on cover slips were rinsed in hot 50% glacial acetic acid for one second and then returned to 50% ethanol at room temperature.

Preparation for and Examination by Scanning Electron Microscopy

Methanol acid prepared chromosomes and chromatid configurations on cover slips were processed through a graded series of ethanol (60, 75, 90, 95 and 100%) followed by two acetone rinses. Specimens were critical point-dried in a Sandri-790 according to standard procedure using liquid carbon dioxide. Selected areas of a cover slip were cut with a diamond pencil and mounted on stubs with silver paint. Specimens were coated with 5–6 nm of gold/palladium with a Technics Hummer Sputter Coater, and then were examined and photographed with an AMR-1000A scanning electron microscope operated at 30 kV. Structures were photographed at 0° to 85° tilting angle, and images were recorded on Polaroid type 55P/N film.

Morphometric Determinations

Morphometric data were obtained using a particle measurement system that was a component of the Alphanumeric Display of the scanning electron microscope. Diameters were determined when a structure was perpendicular to the electron beam, and length was determined by tilting a structure 90° which placed it parallel to the beam. Depth was calculated using either chromatid diameter as an approximate unit and/or with the particle measurement system by tilting structures at 45° followed by rotation through 180°. Other morphometric determinations (Table 1) were made with an Apple II Computer using a purchased Stereometric Measurement and Analysis Program (Scientific Microprograms, Raleigh, N.C.).

Measurements ± standard deviations were obtained from methanol:acetic acid, alcohol dehydrated structures coated with gold/palladium, and they should reflect relative rather than absolute size. However, based upon the diameter of interphase nuclei visualized in this manner (5 to 8 micrometers) compared to the diameter of interphase nuclei visualized in whole cells by phase microscopy (5 to 8 micrometers), our SEM values could be representative of intracellular dimensions.

Results

Isolation and fixation of mitotic chromatid configurations

We employed a methanol acid procedure to both colcemid (Figure 1) and non-colcemid (Figure 2) treated preparations of dividing cells. Primary cultures of peripheral leukocytes and human amniotic cells, and continuous cell lines such as HT-1080, $HeLaS_3$, CHO-K^1, mouse (R-21, LT-10, L-8) and Indian muntjac have yielded well-spread intact mitotic configurations that retained a full intact complement of chromatids. $HeLaS_3$, CHO-K^1, Indian muntjac, and mouse metaphase plates have been collected by selective detachment from both synchronized and unsynchronized cell populations. $HeLaS_3$ was the cell line used primarily in this study.

A majority of the colcemid-treated metaphase plates lost chromosomes during spreading and processing for SEM, whereas the non-colcemid treated metaphase, anaphase and telophase configurations remained intact whether or not the cells (Figures 2,3,4) were previously hypotonically swollen. Hypotonic conditions produced a minimal dispersal effect on non-colcemid prepared metaphases. Prolonged hypotonic swelling (30 minutes or more), however, produced an area at the center of the non-colcemid collected metaphase plates that contained loosely attached chromosomes, producing a plate when viewed from a polar perspective that resembled a ring (Figure 5).

Methanol acid fixed cells remained in fixative for 24 hours prior to final rinsing and collecting for slide preparation. If cells were fixed for less than 24 hours, there was considerable distortion of chromosomal alignment in the configurations when viewed by SEM. If slides were prepared from cells which were in fixative longer than a week, the quality of the configurations was less than optimal. Metaphase plates from non-colcemid treated Indian muntjac cells were bound together (Welter et al., 1984) with a fibrillar network composed of interconnecting fibers of the same diameter (45 nm) as the fibers observed in the connecting fibrillar material of chromosomes in a metaphase plate. In $HeLaS_3$ metaphase configurations, similar fibers were observed extending between hypotonically swollen, adjacent centromeric regions. (Figure 6). Rarely was such interchromosomal material found in metaphase plates after colcemid treatment.

Evaluation of the methanol acid isolation of mitotic configurations for visualization by SEM

Several methods which combine salts and/or detergents were surveyed to determine whether the results, obtained with methanol acid isolated mitotic configurations, represented artifacts or a reasonable facsimile of the natural arrangement of chromosomes and chromatids in metaphase, anaphase and telophase configurations. Each procedure yielded chromatid structures with similar dimensions (Table 1). However, the amount of detail present on the surface of the chromatids varied considerably from one technique to the other. For example, a hexylene glycol procedure of Wray and Stubblefield (1970) yielded metaphase plates (Figure 7) with the same shape, chromosomal size (Table 1) and chromosomal spatial arrangement as the acid isolated metaphases, but the chromosomal surface fine structure was not as detailed, and the configurations observed apparently contained components of the mitotic apparatus such as spindle fibers and the centrosome. The anaphase configurations were likewise obscured by mitotic apparatus, whereas the telophase structures contained small amounts of spindle fibers, patches of nuclear envelope (Figure 8) and some cytoskeletal elements. Mitotic configurations isolated with a detergent procedure followed by sedimentation (Paulson, 1982) resulted in metaphase, anaphase and telophase structures with the same basic shape, chromatid arrangement and size (Table 1) as those isolated with acetic acid (Figure 9). However, these configurations retained no chromatid detail and were contaminated with fragmented mitotic apparatus and some cytoskeletal elements.

Whole cells containing late stages of mitosis collected following synchronization were fixed in 1% glutaraldehyde. An indirect immunostaining was then performed by exposing the cells to serum containing anti-histone antibody and then staining with a mixture of fluorescein-conjugated IgG, IqM and IgA. The stained configurations, revealed by immunofluorescence, closely resembled both living whole mitotic cells seen with phase contrast microscopy and those isolated with the above procedures for SEM (data not shown). Also, whole mitotic cells prepared for TEM revealed chromatid configurations similar to those visualized with SEM and light microscopy.

Effects of Acetic Acid

Intact configurations free of plasma membrane, mitotic apparatus and cytoskeletal elements which exhibit excellent resolution for chromosomal and chromatid structure, clarity of chromatid positioning and arrangement throughout mid to late mitosis were

best obtained with acetic acid isolation. Several factors contribute to the success of this procedure. First, fixation with 3:1 methanol:acetic acid has several advantageous effects in that it does not produce a shrinkage of the configuration, cross-linking of nuclear proteins or loss of structural proteins (Welter and Hodge, in preparation). There is some loss of histones (Ris, 1978) without alteration of chromatid structure which could be helpful in obtaining structures with high resolution. Because of the absence of cross-linking of cytoplasmic proteins, preparations can be repeatedly washed to remove cellular debris and soluble protein, which might otherwise obscure the configurations. Also, 3:1 is the most suitable fixative for spreading of configurations onto a glass surface without the loss of material during the process. Because of the speed of the evaporation of the methanol from the 3:1, one can monitor the rapid settling of the cells onto the glass surface. This can only be done with cells suspended in organic solvents, such as acetone or alcohols which, however, cause shrinkage and distortion of the configurations. Once the configurations have made contact with the glass, there is an affinity which permits further rinsing with 50% acetic acid at 100°C. This brief rinsing with the hot acid achieves several objectives which contribute to the success of this procedure. First, the hot acid removes remaining membranes from the preparation. Also, acetic acid in the 3:1 mixture swells configurations evenly so that the brief exposure to 50% hot acid, flushes away labile proteins, and organelles in the rinse. The swelling phenomenon can be calculated if the preparation is permitted to air dry following the hot acid rinse. However, if the slides are immersed in 50% ethanol (at room temperature) immediately following the hot acid rinse, the configurations shrink back to their normal size free of contaminants. The hot acid can have an adverse effect on the configuration if the preparations are exposed too long. After several seconds exposure to 50% acetic acid at 100°C, chromosomes and chromatids begin to erode resulting in configurations composed of a delicate framework of thin fibers, somewhat resembling a configuration treated with high molar salts or urea (personal observation). Several minutes exposure to the hot acid reduces the configurations to skeletons of "melted" chromosomes (Figure 10). Temperatures below 95°C will remove membranes and cytoplasmic organelles with increased exposure (time), all at the expense of chromosomal morphology. Acetic acid concentrations below 40% are ineffective in producing the effect necessary to "clean" structures, and concentrations above 65% at 100°C damage the mitotic structures.

Dramatic changes in chromosome morphology and metaphase plate configuration can be produced by varying the staging solution following hot acid treatment. 50% ethanol at room temperature produced the most desired effect. Metaphase plate and chromosome morphology were retained when cells were stationed in 50% ethanol for 10–30 minutes. There was little difference in quality of chromosomes and/or configurations when 40–70% ethanol was used as an initial stop in dehydration. However, if the concentration of this initial ethanol solution was below 40%, surface appearance of chromosomes became irregular with a wide variation in fiber diameter (20–100 nm). This same effect was seen when chromosomes were stationed in water, phosphate buffered saline or other fixatives following the hot acid rinse. When cells were stationed in 1% glutaraldehyde directly from the hot acid, surface morphology was decreased, but when cells were briefly fixed in 50% ethanol (for 10 min)

CAPTIONS:

Figure 1. Metaphase "plate" from colcemid-arrested human amniotic primary cells. This metaphase plate was visualized from cells which were fixed for 24 hours in 3:1 methanol:glacial acetic acid and rinsed for two seconds with 50% acetic acid at 100°C. Following this acid rinse, preparations were dehydrated in ethanol and critical point dried. Bar = 5 μm.

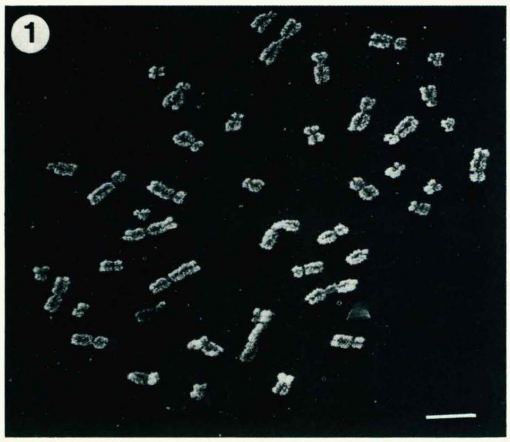
Figure 2. Metaphase plate collected following a double thymidine blockade of HeLa cells. Note that the longer chromosomes, J-and-V shaped, assume a peripheral position, unlike the random arrangement of a colcemid arrested metaphase in Figure 1. Cell preparations, as in Figure 1, were hypotonically swollen for five minutes. Following 24 hours fixation in methanol acetic acid, this preparation was rinsed in hot 50% acetic acid before dehydration in ethanol, and critical point drying. Bar = 5 μm. H – homologous chromatids, C – centromeres.

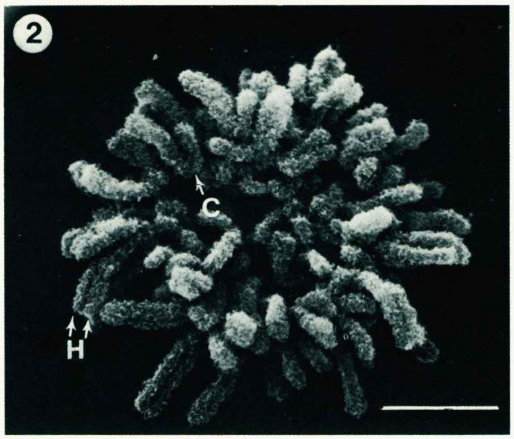
Figure 3. Mid-anaphase chromatid configuration collected by shake-off from a culture of diploid primary human cells. Cells were hypotonically swollen, fixed in 3:1 methanol acetic acid, rinsed in hot 50% acetic acid for one second, and dehydrated through graded ethanol series before critical point drying. The configuration is free of organelles, mitotic apparatus and membranes. Occasionally small vesicles (V), thought to be vesticulated membrane, remained attached. Bar = 2 μm.

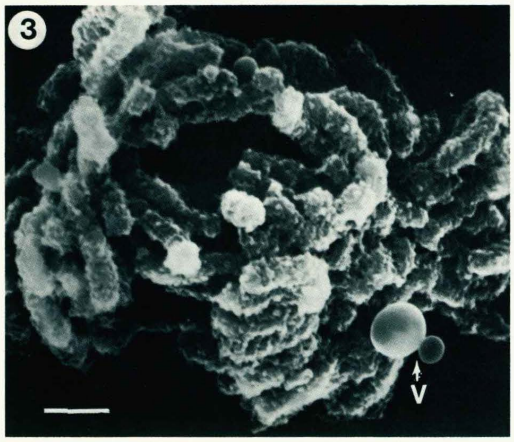
Figure 4. An attached pair of telophase chromatid structures. This pair of telophase structures remains attached by a peduncle (P) of unknown origin. It is conceivable that the peduncle represents the midbody. Note that this configuration has a smaller diameter (approximately 8 μm) than the metaphase and anaphase configurations. Flexed regions of the longer, peripherally placed chromatids form the rim (R) surrounding the opening of the chamber which is characteristic for early telophase in $HeLaS_3$ cells. Bar = 5 μm.

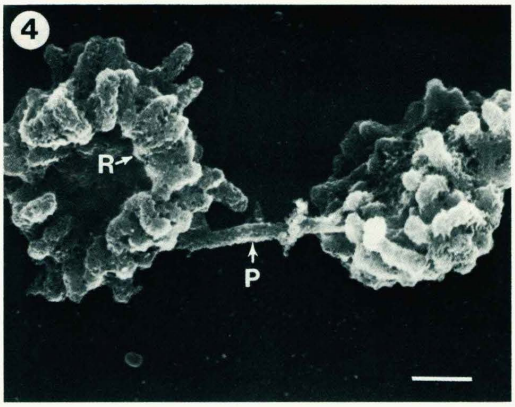
Figure 5. Hypotonically spread chromosomes in a metaphase plate. This $HeLaS_3$ metaphase was prepared by exposing the collected cells to hypotonic condition for 30 minutes before fixation with 3:1 methanol:acetic acid. Chromosomes retain their spatial arrangement, but the smaller, centrally located chromosomes appear to have lost their interchromosomal or interconnecting fibers leaving the center of the configuration sparsely filled. Close observation of the peripheral chromosomes reveals interconnecting fibers between adjacent centromeres. Bar = 10 μm.

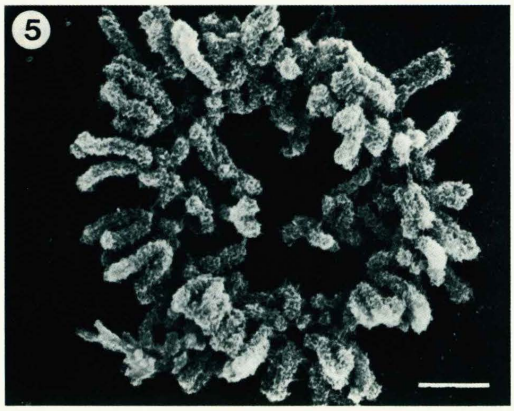
Figure 6. $HeLaS_3$ peripheral chromosomal interconnecting fibers (IF) exposed by excessive hypotonic swelling. This metaphase plate was prepared by exposing the collected cells to hypotonic conditions for 60 minutes before routine acid alcohol fixation. Metaphase chromosomes retain the characteristic 45 nm fiber found with acetic acid prepared chromosomes. Interconnecting fibers are also 45 nm structures. Bar = 0.5 μm.

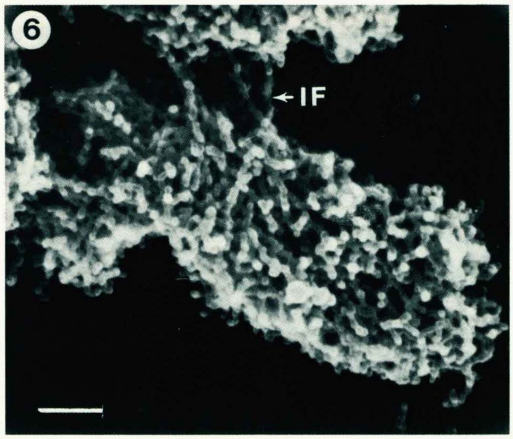

1


2
C
H


3
V


4
R
P


5


6
IF

following 30 minutes in glutaraldehyde surface morphology was enhanced (Figure 11). Gallocyanin chromalum (1% at room temperature for 1 hour) will reduce the length of the chromosomes by 30% and increase the diameter of the fiber from 45 nm to 90–100 nm (Figure 12) when stationed directly from the hot acetic acid, while changes are not so drastic when the preparation is fixed after 10 min in 50% ethanol. Other fixations, such as 10% formalin, methanol:acetic acid:formalin, periodate-lysine-paraformaldehyde and osmium tetroxide-thiocarbohydrazide directly or following 10 min 50% ethanol failed to produce chromosomes with sufficient detail.

Initial fixation of mitotic cells with any fixative other than 3:1, 2:1 or 1:1 methanol:acetic acid, 3:1 methanol:formic acid or 50% acetic acid fixes cytoplasmic structures and membranes such that removal of these cellular components by shearing forces with the hot acetic acid was inhibited. We have found that 24 hours fixation after collection of cells, stabilizes the mitotic cells, so that random dispersal of chromosomes does not occur, and a greater percentage of intact metaphase are retained in the preparation of slides for SEM.

Discussion

The purpose of these results and this discussion was not to describe specific chromatid and chromosomal arrangements into configurations which are quite distinct for $HeLaS_3$ cells at metaphase, early anaphase, mid anaphase, late anaphase and telophase; but was to demonstrate that a procedure, regarded by some as harsh, enables us to three-dimensionally visualize with SEM, these configurations, far better, than those isolated with established techniques (Wray and Stubblefield, 1970; Paulson, 1982). The SEM description of the late mitotic events in $HeLaS_3$ and the unique alignment of anaphase chromatids in $HeLaS_3$ as visualized by SEM will be described elsewhere (Welter et al., in preparation).

With regard to the harshness of the hot acid rinse on configurations, it was concluded that there was little effect on chromatid arrangement or morphology of configurations fixed in acid alcohol.The morphometric parameters of all configurations are essentially the same (Table 1) whether isolated at near neutral pH in a simple buffer (Wray and Stubblefield, 1970), or isolated with non-ionic buffer at neutral pH (Paulson, 1982), or isolated with hot acid. Live cells visualized with phase microscopy, whole cells prepared for TEM and whole cells visualized after preparation for indirect immunofluorescence, all have configurations which resemble those isolated for SEM.

There is the possibility that acid alcohol fixation produces numerous artifacts. Chromosomal interconnections, real or artifact,have been a topic of discussion for some time (Bahr and Engler, 1980; Korf and Dracumakos, 1980; Paulson, 1982). Chromosomal interconnecting fibers are demonstrated in chromatid configurations isolated with the acid procedure described in this paper and with the methods of Wray and Stubblefield (1970) and Paulson (1982). These fibers are difficult to demonstrate in the $HeLaS_3$ configurations because of the large number of chromosomes (approximately 65). However, these fibers increase in number as mitosis progresses from metaphase to telophase. Specific 45 nm fibers interconnecting adjacent centromeric regions of the Indian muntjac have been described by Welter et al. (1984).

CAPTIONS:

Figure 7. Diploid human primary cell anaphase isolated by the hexylene glycol method of Wray and Stubblefield (1970). Note that the centriole (CT) and spindle tubules (T) apparently remain attached to the anaphase chromatid configuration following this isolation procedure. Shape, size and chromatid arrangement is indistinguishable from the anaphase configuration isolated by the acid alcohol procedure (see Figure 3). Bar = 1 μm.

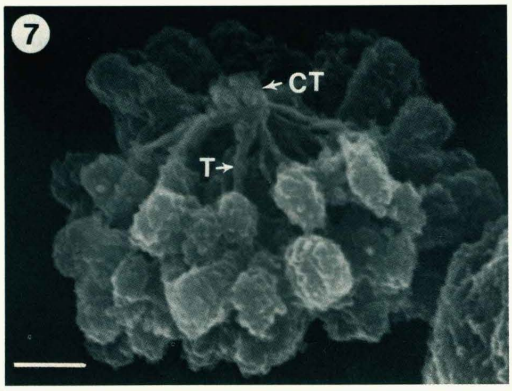
Figure 8. Early telophase configuration from a diploid human primary cell line isolated with hexylene glycol. The spindle apparatus appears to be intact, and newly formed patches of membrane (M) appear to be on the surface of some of the chromatids. Bar = 1 μm.

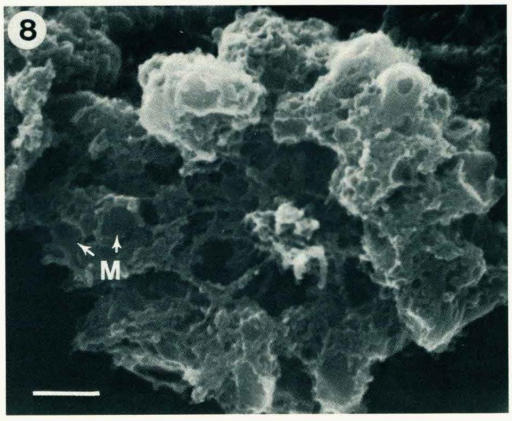
Figure 9. Diploid primary cell telophase configuration isolated with a non-ionic detergent method of Paulson (1982). Cytoskeletal fragments (FG) cover the surface of the chromatids, and remnants of the mitotic spindle occupy the chamber. Bar = 1 μm.

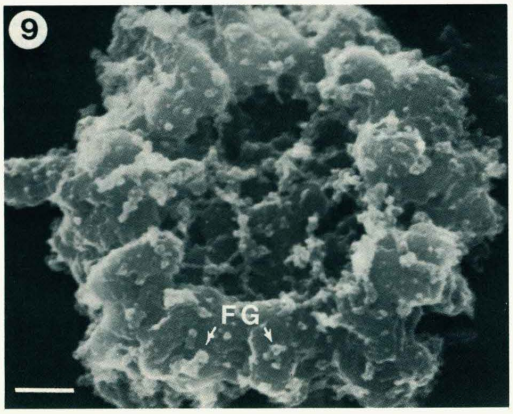
Figure 10. $HeLaS_3$ pro-metaphase chromosomes collected from colcemid arrested cultures. Cells were prepared as described in figure 1 except that they were rinsed in 50% acetic acid for 60 seconds before dehydration in a graded ethanol series and critical point dried. Chromosomes are severely eroded, leaving only what appears to be a melted skeleton. Bar = 5 μm.

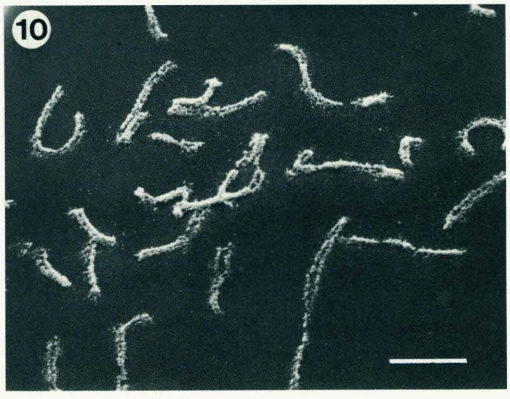
Figure 11. Isolated metaphase chromosome collected from a colcemid arrested culture. $HeLaS_3$ were fixed in 3:1, and rinsed in 50% acetic acid at 100°C for 1 second. Preparations then were stationed in 1% glutaraldehyde for 10 minutes following the hot acid rinse before ethanol dehydration and critical point drying. This chromosome appears to have retained excellent morphology compared to photomicrographs published by others. Bar = 1 μm.

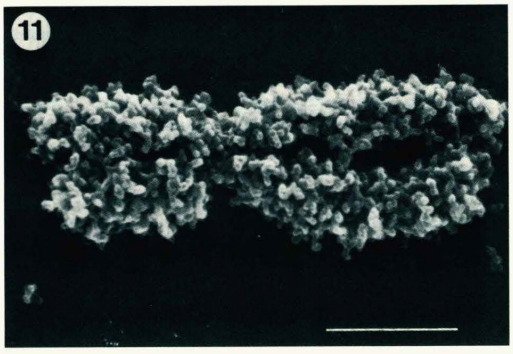
Figure 12. $HeLaS_3$ metacentric chromosome collected from colcemid arrested culture. Cells were fixed in 3:1 methanol: glacial acetic acid, rinsed in 50% acetic acid, stationed in 1% gallocyanin chromalum for 30 minutes before ethanol dehydration and critical point drying. Note the 45 nm fibers now appear to be cross-linked into fewer, but larger 70–100 nm fibers. Bar + 1 μm.

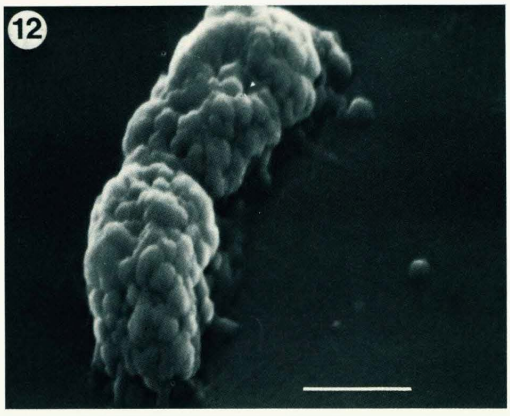

7
CT
T


8
M


9
FG


10


11


12

The most obvious advantage of this technique over the others described is that the morphology of chromosomes of the metaphase, anaphase and telophase configurations is superior (Figure 11). Secondly, acid isolated chromatid configurations are always free of membranes and cytoskeletal elements, and finally the speed of and simplicity in obtaining good preparations can be achieved routinely.

References

Arrighi FE, Hsu TC. (1974). Staining constitutive heterochromatin and Giemsa crossband of mammalian chromosomes. In: Human chromosome methodology. JJ Yunis (Ed.), New York and London, Academic Press, Ch. 4., pp. 59–71.

Bahr GF, Engler WF. (1980). Chromosome interconnections. A discussion of their reality. Cancer Genet. Cytogent. **1:** 177–185.

Barnett RI, MacKinnon EA, Romero-Sierra C. (1974). The lability of acid-alcohol fixed human chromosomes. Cytobios **11:** 115–123.

Barnicott NA, Huxley HE. (1961). The electron microscopy of unsectioned human chromosomes. Ann. Hum. Genet. Lond. **25:** 253–258.

Black DA, Welter DA, Tischfield JA, Hodge LD. (1984). The alignment of chromatids during late mitosis in human cells. J. Cell. Biol. **99:** 448a.

Burkholder GD. (1974). Electron microscopic visualization of chromosomes banded with trypsin. Nature **247:** 292–294.

Emmerich G, Scharrer ZS, Stengel-Rutkowski S, Zang KD. (1973). Elektronenmikroskopische Untersuchung interchromosomaler fadiger strukturen an meschlichen lymphocyten in der metaphase. Humangenetik **19:** 227–234.

Felluga B, Martinucci GB. (1976). A simple method for karyotyping by transmission electron microscopy. J. Submicrosc. Cytol. **8:** 347–352.

Ford EHR, Thurley K, Wollam DHM. (1968). Electron-microscopic observations on whole human chromosomes. J. Anat. **103:** 143–150.

Golomb HM, Bahr GF. (1971). Scanning electron microscopic observation of surface structure of isolated human chromosomes. Science **171:** 1024–1026.

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.

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

Heneen WK. (1981). Mitosis as discerned in the scanning electron microscope. Europ. J. Cell Biol. **25:** 242–247.

Ip W, Fischman DA. (1979). High resolution scanning electron microscopy of isolated and *in situ* cytoskeletal elements. J. Cell Biol. **83:** 249–254.

Johnson PW, Barnett RI, MacKinnon EA. (1982). Image analysis of the acetic acid-alcohol fixed metaphase chromosome. J. Submicrosc. Cytol. **14:** 31–43.

Kalisch WE, Jacob HJ. (1983). Scanning electron microscopy of surface spread polytene chromosomes. Cytobios **36:** 39–43.

Katsumoto T, Naguro TI, Iino A, Takagi A. (1981). The effect of tannic acid on the preservation of tissue culture cells for scanning electron microscopy. J. Electron Microsc. **30:** 177–182.

Korf BR, Dracumakos EG. (1980). Absence of true interchromosomal connectives in microsurgically isolated chromosomes. Exp. Cell Res. **130:** 377–385.

Laane MM, Wahlstrom R, Mellem TR. (1977). Scanning electron microscopy of nuclear division stage in *Vicia faba* and *Haemanthus cinnabarinus*. Hereditas **86:** 171–178.

Leick V, Bro BI, Lima-De-Faria A. (1979). Scanning electron microscopy of the nuclei and nucleoli in Tetrahymena. Hereditas **90:** 219–226.

Lubit BW, Pham TD, Miller OJ, Erlanger BF. (1976). Localization of 5-methylcytosine in human metaphase chromosomes by immunoelectron microscopy. Cell **9:** 503–509.

Neurath PW, Ampola MG, Vetter HG. (1967). Scanning electron microscopy of chromosomes. Lancet **11:** 1266–1267.

Paulson JR. (1982). Isolation of chromosome clusters from metaphase-arrested HeLa cells. Chromosome **85:** 571–581.

Ris H. (1978). Preparation of chromatin and chromosomes for electron microscopy. Methods of Cell Biology, Vol 18, G. Stein, J. Stein, L.J. Kleinsmith (Eds.), Acad. Press, NY, pp. 229–246.

Ris H. (1979). Ultrastructure and molecular organization of genetic systems. Can. J. Genet. Cytol. **3:** 95–120.

Ruzicka F. (1971). A simple method for the preparation of human metaphase chromosomes for electron microscopy. Humangenetik. **13:** 199–204.

Simmons T, Heywood P, Hodge LD. (1973). Nuclear envelope associated resumption of RNA synthesis in late mitosis of HeLa cells. J. Cell. Biol. **59:** 150–164.

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

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 **86:** 683–702.

Vincent RA, Gilbert LM, Doty SB, Mertz T. (1975). A blaze-dry spreading procedure for the electron microscopy of chromosomes from acid alcohol-fixed human leukocytes. Stain Technol. **50:** 233–237.

Welter DA, Scholer J, Rosenquist TH. (1979). Gallocyanin-chromalum for improved scanning electron microscopy of whole nuclei without critical point drying. Stain Technology **53:** 311–314.

Welter DA, Henry S, Hodge LD. (1982). An examination by SEM of chromosomal behavior during nuclear reformation in HeLa-S_3 cells. J. Cell Biol. **95:** 85a.

Welter DA, Black DA, Ulmer T, Hodge LD. (1983). Anaphase alignment in HeLa-S_3 cells is bi-telomeric which is not consistent with a Rabl Orientation. J. Cell Biol. **97:** 188a.

Welter DA, Black DA, Hodge LD. (1984). Chromosome stabilizing structures in mitotic Indian muntjac (*Muntiacus muntjak*) cells. Experientia **40:** 871–873.

Wray W, Stubblefield TE. (1970). A new method for the rapid isolation of chromosomes, mitotic apparatus, or nuclei from mammalian fibroblasts at near neutral pH. Exp. Cell Res. **59:** 469–478.

Discussion with Reviewers

A.T. Sumner: Although the authors' results are very satisfying aesthetically, there seems little doubt that methanol-acetic acid fixation and hot acetic acid treatment remove substantial amounts of protein and probably damage DNA, (e.g. Brody 1974, Exp. Cell Res. **85:** 255–263; Burkholder and Duczek 1980, Chromosoma **79:** 29–41). It may therefore be that the results obtained by the methods of Paulson and of Wray and Stubblefield are actually more life-like. The authors admit that excessive acetic acid treatment severely damages the chromosomes. Would the authors please comment, and perhaps enlarge upon their statement in the text that there is no loss of structural proteins?
Authors: We discussed the results obtained with two other isolation procedures, and it is clear that the basic morphological appearance and dimensions of chromatid structures are indistinguishable. Only the methanol-acid fixation followed by hot acid permitted a detailed visualization of chromatid alignment and orientation. It is likely that DNA has been damaged, however, it appears unlikely that DNA integrity is essential for the isolation of mitotic chromatid configurations. For example, digestion of greater than 75% of the DNA with deoxyribonuclease, based upon the recovery of radioactivity after prelabeling with [$^{+3}$H]-thymidine, did not alter morphology or morphometric values. As has been demonstrated with isolated metaphase chromosomes, structural integrity appears to be independent DNA content. Also we have observed that high salt and urea extractions yield easily recognized "skeletons" of each configuration, suggesting that protein:protein interactions necessary for structural integrity are not removed by our procedure. Based on the recovery of histones separated in one-dimensional acrylamide gels, we have removed 20% to 30% of these components.

A.T. Sumner: What holds the chromosomes together when all the other cellular constituents have been removed?
Authors: We have morphologic evidence that there are interchromosome and interchromatid fibers that maintain structural integrity. We would also hypothesize that based on other observations with salt and urea extractions (previously mentioned in the answer to your first question) that there could be a skeletal structure to the entire chromatid configuration, components of which could extend through such fibers. We have also observed a centromeric ring of similar appearing fibers in Indian muntjac cells which we recently published. (Welter et al., 1984).

T.D. Allen: When the resuspended pellet was applied to the coverslip, did complete air drying (i.e., the passage of a liquid vapour interface across the preparations) occur? If not, how were the metaphases released from the albeit swollen, but probably still intact membrane and cytoplasm?
Authors: Complete air drying did not occur to the cells applied to the coverslip. However the methanol portion of the fixation evaporated permitting the cells to make contact with the glass, prior to rinsing with hot acetic acid. The release of the chromosomes and configurations from the intact membrane and cytoplasma component was achieved by solubilizing the membrane lipids. The hot acid produces excessive swelling which can be demonstrated by permitting the cells to air dry. This excessive swelling releases the configuration from the cellular organelles and membrane.

T.D. Allen: What is the fibre diameter of the chromatin in the chromosome in Fig. 11? It appears to be more of the diameter of the typical Wray and Stubblefield extraction method (i.e., 45 nm as per Fig. 6). It has been our experience that when normal unburst 3.1 fixed metaphases are re-pelleted and subsequently prepared for TEM, the fibre diameter is more like the 25–30 nm of the typical low pH extracted chromosomes. Does the hot acid treatment then produce a swelling of the chromatin fibre?
Authors: The fiber diameter of the chromosomes in Figure 11 is approximately 38 nm along its length and the knob at the distal end (periphery of the chromatid) is approximately 43 nm. The hot acid presumably increases fiber diameter because other authors have reported the 25–30 nm diameter in low pH extracted chromosomes.

T.D. Allen: Have the authors observed any variation in either chromosome length or chromatin packing when comparing non-colcemid metaphases with colcemid accumulated metaphases?
Authors: It is known that chromosomes in the presence of colcemid become more tightly coiled or compacted with increasing time of exposure to the drug. It would then be expected that the length of chromatids in non-disrupted metaphase plates would be longer than those in colcemid disrupted plates. However, we have not tried to make this measurement and suspect that this would be rather difficult because of the number of chromosomes in $HeLaS_3$ cells and the difficulty in identifying specific chromosomes due to their crowding in non-disrupted plates.

K.R. Utsumi: Were the cells spread over the cover slip completely dried, or were they still wet with the fixative, when they were rinsed in hot 50% glacial acetic acid? Did they dry up in 5 sec?
Authors: The cells were still wet when they were rinsed in hot 50% glacial acetic acid. Cells spread on cover slips were visually monitored in bright light for the evaporation of the fixative. When the cells make contact with the glass presumably most of the methanol has evaporated thereby resulting in a "grainy" appearance of the preparation. The cells are still wet at this time and are immediately immersed in hot acid. The time for evaporation of the methanol from the fixative varies from 5 to 30 seconds depending on the atmospheric conditions. This is the importance of monitoring the evaporation rate so that the cells do not dry.

K.R. Utsumi: Surface topology of a chromosome shown in Fig. 12 must be an artifact caused by coating with Au/Pd, a decoration effect. Or is it a result due only to gallocyanin chromalum?
Authors: The surface morphology is not an artifact because gallocyanin chromalum chromosomes do not require Au/Pd coating for visualization. There is sufficient conductivity of chromosomes fixed in gallocyanin chromalum to visualize them uncoated, and the appearance is the same with the Au/Pd coated material or uncoated material.

K.W. Adolph: It is well documented that acid fixation of chromosomes can extract proteins, particularly the histones.Can the authors estimate the amount of protein extracted in their preparations? What is the evidence that this extraction does not affect chromatid structure?

Authors: Compared to detergent isolations, we know that 20 to 30% of the histones are extracted. We have not made a systematic survey of the retention of non-histone proteins, although we do know, based on immunofluorescence using monoclonal antibody supplied by Dr. Gerd Maul, that at least some of the lamins are not extracted. Also if nuclei are isolated from interphase cells by methanol acid or by detergents, methanol-acid nuclei contain 70 to 80% of the protein of detergent-cleaned nuclei, suggesting that a major portion of the chromatid protein should be preserved in the configurations which we have visualized.

Concerning the basic structure or organizations of an individual chromatid, we have no information about the effect of our isolation. Our data argue that the spatial arrangement between chromatids has been preserved, thus permitting insight into their alignment during nuclear reformation.

K.W. Adolph: What general conclusions can be made regarding changes in the three-dimensional arrangement of chromosomes from metaphase to anaphase to telophase?

Authors: We have in press (Chromosoma) our analysis of changes in the $HeLaS_3$ cells. It appears, for example, that a cylindrical alignment at anaphase consists of a closed base composed of the shortest chromatids joined along their lateral length. The sides are composed of a double concentric layer of the longest chromatids which are joined progressively along their lateral length during anaphase. This process begins at the centromere working its way toward the telomeres. By early telophase a configuration resembling a hollow half sphere is formed. There is loss in depth of the chamber of their structure which involves an apparent flexing of chromatids. Only after loss in chromatid outline is there chromatin decondensation. Initial data with Indian muntjac cells indicate significant differences in this mammalian cell with regard to chromatid behavior during nuclear reformation.

K.W. Adolph: Do the authors believe that most chromosomes have interconnective fibers (Fig. 6)? On average, how many extend between different chromosomes?

Authors: We do believe chromosomes at metaphase have interconnecting fibers such that two interconnected sets of chromatids exist joined at the centromere. It is tempting to speculate even further with the suggestion that such fibers maintain the nonrandom positions of chromosomes in a metaphase plate resulting in the proper position of chromatids for subsequent nuclear reformation which is initiated as an early anaphase event.

At present spreading conditions using hypotonic swelling are unsatisfactory to permit an estimate of the number of fibers between adjacent chromosomes. Conceivably, mammalian cells with a low number of chromosomes and/or thick sections for transmission electron microscopy would allow such an estimate.

K.W. Adolph: How frequently observed was the "peduncle" shown in Fig. 4 and the characteristic chamber of early telophase?

Authors: The peduncle, or interconnection measured between sister telophase configuration is found in less than five percent of the telophase structures scored. The peduncle is characteristic of $HeLaS_3$ and not observed in other mammalian cell lines we have surveyed. The characteristic chamber observed in early telophase is observed in all cell lines at this state of nuclear reformation.