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Spatial arrangement of homologous chromosomes during anaphase in early embryos of *Drosophila melanogaster* studied by three-dimensional fluorescence microscopy

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Abstract. Using a computer-controlled fluorescence microscope system, spatial arrangement of homologous chromosomes was analyzed in fixed and living embryos of *Drosophila melanogaster* at the syncytial blastoderm stage. In fixed embryos, chromosomes were stained with a DNA-specific fluorescent dye, 4′,6-diamidino-2-phenylindole; the arrangement of anaphase chromosomes was determined with their identification by high-resolution analysis. In living embryos, the behavior of chromosomes during anaphase was examined by the use of a strain carrying a long translocated chromosome as a cytological marker to identify the chromosome while the chromosomes were stained by microinjection of rhodamine-conjugated histones into the embryos. Chromosome arrangement was also examined in nuclei that had swollen artificially under anoxic conditions for better spatial separation of individual chromosomes in such nuclei. All these experiments consistently showed that homologous chromosomes were not associated with each other in syncytial blastoderm embryos of *Drosophila melanogaster*. Our studies also demonstrated that cytological tools greatly facilitate the dissection of nuclear structures when used in combination with imaging technology.

Keywords: three-dimensional fluorescence microscopy, time-lapse microscopy, computational image processing, homologous chromosome, anaphase, *Drosophila* embryo

1. Introduction

The existence of an ordered arrangement of homologous chromosomes in diploid nuclei has been debated for nearly a century. Cytological observations have inferred somatic pairing of homologous chromosomes in many organisms; homologous chromosomes are often found close to each other in spread preparations of metaphase chromosomes (reviewed in Avivi and Feldman 1980, Comming 1980, Fussel 1984, Hilliker and Appels 1989). Statistical models have also been proposed to reconstruct the original three-dimensional positions of chromosomes from their two-dimensional spread (Lacadena and Ferrer 1978). Alternatively, direct three-dimensional reconstruction from

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serial electron microscopy sections has been used to determine the arrangement of metaphase chromosomes (Kubai 1987, Mosgoller *et al* 1991, Leitch *et al* 1994). In the last decade, the development of three-dimensional imaging technology in light microscopy, such as confocal microscopy (reviewed in Wilson 1990) or computational image processing methods (Fay *et al* 1989, Agard *et al* 1989), has made it possible to directly determine spatial organization of chromosomes within an intact nucleus on a routine basis.

In addition to the development of microscope technology, a variety of methods for fluorescence staining of chromosomes have been developed: for example, the so-called chromosome painting by *in situ* hybridization using chromosome-specific DNA probes to determine the nuclear location of interphase chromosomes as well as to identify mitotic chromosomes (e.g. Lichter *et al* 1988, Cremer *et al* 1988, Pinkel *et al* 1988, Lengauer *et al* 1990, Ried *et al* 1992, Trask *et al* 1993). By using the combination of chromosome-specific painting probes with multi-spectral fluorescent dyes, it is even now possible to distinguish all human chromosomes simultaneously (Speicher *et al* 1996a, b, Schröck *et al* 1996, Garini *et al* 1996). Made possible by these technological developments, the spatial arrangement of chromosomes within a nucleus has recently been extensively studied in mammalian tissues and culture cells in which the most extensive collection of DNA probes are available; the functional organization of chromosomes within a nucleus has been demonstrated in relation to cellular events (Manuelidis 1990, Cremer *et al* 1993, Strouboulis and Wolffe 1996). Cytological observations have revealed that individual chromosomes occupy discrete territories in interphase nuclei. Homologous chromosome territories are separate from each other in many cases (Manuelidis and Borden 1988, Popp *et al* 1990, Kurz *et al* 1996, Eils *et al* 1996), and in other cases, a specific pair of homologous chromosomes are found in close apposition in certain tissues, cell types or cell cycle stages (Arnoldus *et al* 1989, Lewis *et al* 1993, LaSalle and Lalande 1996).

In *Drosophila*, direct microscopic evidence of homologous chromosome synapsis has been obtained in specialized tissues containing polytenized giant chromosomes. In polytene nuclei, the physical association of homologous chromosomes along their entire length can clearly be seen (Ashburner 1989). In addition, it has been assumed that homologous chromosomes in *Drosophila melanogaster* are physically associated in diploid somatic nuclei, as genetic evidence has shown that the expression of several genes can be affected by the pairing state of the homologous locus. These genetic effects, known as transvection, seem to depend on trans interactions between homologous sequences; chromosomal translocations disrupting the proximity of homologous chromosomes eliminate the transvection effect (reviewed in Wu and Goldberg 1989, Pirrota 1990). These observations imply a role of the spatial location of chromosomes for gene expression in differentiated tissues.

In order to determine whether the spatial arrangement of chromosomes in undifferentiated cells is similar to or different from that in differentiated tissues, we have examined the spatial arrangement of chromosomes in syncytial blastoderm embryos. Embryos at the 10th to 13th nuclear division cycles provide an attractive experimental system for microscopic observation of the arrangement and dynamics of chromosomes. At this stage of embryonic development, several thousand nuclei form a monolayer immediately below the surface of the embryo, dividing synchronously every 10–20 min within a single cytoplasm (Zalokar and Erk 1976). After the 13th nuclear division, embryos start the process of differentiation with asynchronous mitoses; only a few more cycles of mitosis allow further embryo differentiation (Foe 1989). Thus, in *Drosophila* embryos nuclear structures can be examined during the mitotic cell cycle as well as during the process of differentiation.

We then exploited these advantages of *Drosophila* embryos to examine the arrangement of chromosomes during the mitotic cell cycle. This was accomplished by the use of high-resolution three-dimensional light microscopy in conjunction with computational image processing (Agard *et al* 1989, Hiraoka *et al* 1991). During prophase and metaphase when chromosomes are condensed, arrangement of chromosomes was determined by tracing their three-dimensional paths along the entire length (Chen *et al* 1989, Hiraoka *et al* 1990a); individual chromosomes were distinguished by their characteristic staining pattern at the centromeric heterochromatin at prophase whereas they could not be identified at metaphase because centromeric regions were closely packed in the metaphase plate. Results based on traced paths of chromosomes at prophase and metaphase indicate that homologous chromosomes are not synapsed in syncytial blastoderm stage embryos (Hiraoka *et al* 1990a). In contrast, when chromosomes were decondensed in interphase, the location of homologous chromosomes was examined by fluorescence *in situ* hybridization to a specific chromosomal locus. Experiments of *in situ* hybridization showed that homologous loci of histone genes were not associated with each other (Hiraoka *et al* 1993). Unlike the cases of human nuclei, however, the domains of entire chromosomes in interphase nuclei have not been demonstrated in *Drosophila*. In this paper, we describe the arrangement of anaphase chromosomes examined by the path-tracing analysis in fixed specimens. Here we also demonstrate the use of cytological tools available in *Drosophila*. We examined the chromosome arrangement of nuclei in which chromosome condensation had been induced by keeping the embryos under anoxic conditions; the chromosome condensation caused by anoxia facilitated the path-tracing in early prophase. Results obtained from fixed specimens

were further confirmed by the live analysis of living embryos using a mutant strain bearing a translocated long chromosome arm. These results, combined with the previous studies (Hiraoka *et al* 1990a, 1993), lead consistently to the conclusion that a homologous pair of chromosomes are not in close apposition with each other, but rather in random arrangement, throughout the mitotic cycle at this early developmental stage of *Drosophila* embryos.

2. Materials and methods

2.1. Microscopy image acquisition, processing and analysis

Our computerized microscope system was composed of a fluorescence microscope, a cooled charge-coupled device (CCD) as a high-sensitivity image detector, and a computer that controlled the microscope and the CCD. There were several historical levels of technological development in the configuration of the system. In the early stages of this study, a Peltier-cooled CCD camera (CH250, Photometrics Ltd, Tucson, Arizona) with a 1340 × 1037 pixel CCD chip (KAF1400) was coupled to an Olympus inverted microscope IMT-2; microscope lamp shutter, focus movement, CCD data collection and filter combinations were controlled by a MicroVax III workstation (Hiraoka *et al* 1991). Image processing was carried out on a VAX8650 computer or attached FPS 264 array processor (Floating Point Systems, Beaverton, Oregon). Modeling and image display were carried out on a Parallax model 1280 graphic display with a 12 Mbyte image memory (Parallax Graphics Inc, Santa Clara, California).

More recently, the VAX computer-based system was replaced by a Silicon Graphics UNIX workstation. In the current system configuration, a Peltier-cooled CCD camera with a 1340 × 1037 pixel CCD chip (PXL1400, Photometrics Ltd, Tucson, Arizona) is coupled to an Olympus inverted microscope IMT2 or IX-70. Illumination from the mercury arc lamp is transmitted through an optical fiber to provide a uniform illumination (Kam *et al* 1993); the microscope lamp shutter, neutral density filters, excitation filters, emission filters, *xyz*-motorized microscope stage and CCD data collection are all controlled by a Silicon Graphics workstation. Image acquisition, processing, analysis and display can be carried out on a single workstation using menu-driven graphic software (Chen *et al* 1996). The entire microscope system, complete with software package is now commercially available as DeltaVision (Applied Precision, Inc, Seattle, Washington).

2.2. Optical sectioning microscopy of chromosomes in fixed embryos

Drosophila embryos were fixed with 3.7% formaldehyde (freshly prepared from paraformaldehyde) in a 1:1 mixture of heptane/buffer A (15 mM Pipes pH 7.0, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 0.1% 2-mercaptoethanol). Chorion and vitelline membrane were removed as previously described (Mitchison and Sedat 1983). Anoxic conditions were achieved by keeping embryos in an air-tight vial containing degassed 150 mM NaCl solution overlayed with argon gas for 30 min before fixation. The embryos were stained with 0.1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI). Optical section images were collected on the CCD at focus steps of 0.2 μm or 0.25 μm using an Olympus oil-immersion objective lens (60×/NA = 1.4). The CCD pixel size corresponded to 0.07 μm in the specimen plane. The out-of-focus information was removed by a constrained, three-dimensional iterative deconvolution method using the optical transfer function experimentally determined for the objective lens used (Agard *et al* 1989, Hiraoka *et al* 1990b).

2.3. Computational chromosome painting

Paths of chromosomes were traced by the use of an interactive computer program in a high-resolution, three-dimensional data stack, computationally processed to improve the microscopic resolution. Details of an interactive modeling program to trace chromosome paths in a three-dimensional data stack were previously described (Chen *et al* 1989, 1996). Three-dimensional paths along the center of chromosome fibers were traced by a cursor, while the multiple images from various view angles were updated simultaneously to follow the cursor movement, and recorded as sets of three-dimensional coordinates. Although the central path of chromosomes could be traced, the chromosome surface was not always clearly separated along the focal direction. For this reason, the automated assignment of chromosomes was not successful, so instead we traced and identified each of the individual chromosomes in a fully interactive manner.

Each of the traced chromosomes was identified by the characteristic DAPI-staining pattern (Hiraoka *et al* 1990a). Grey level information associated with the selected chromosome paths was discriminated by thresholding the grey level intensity and/or detecting chromosome edges based on the intensity gradient, and dissected from the rest of the chromosomes; the dissected chromosomes were recomposed as the outlines filled with a particular color (cf figure 3 in Hiraoka *et al* 1990a).

2.4. Optical sectioning microscopy of chromosomes in living embryos

A *Drosophila* mutant strain ltx13 (Wakimoto and Hearn 1990) was obtained from Dr Barbara Wakimoto (University of Washington, Seattle, WA). Rhodamine-labeled calf thymus histones H2A and H2B (a gift of Dr J S Minden)

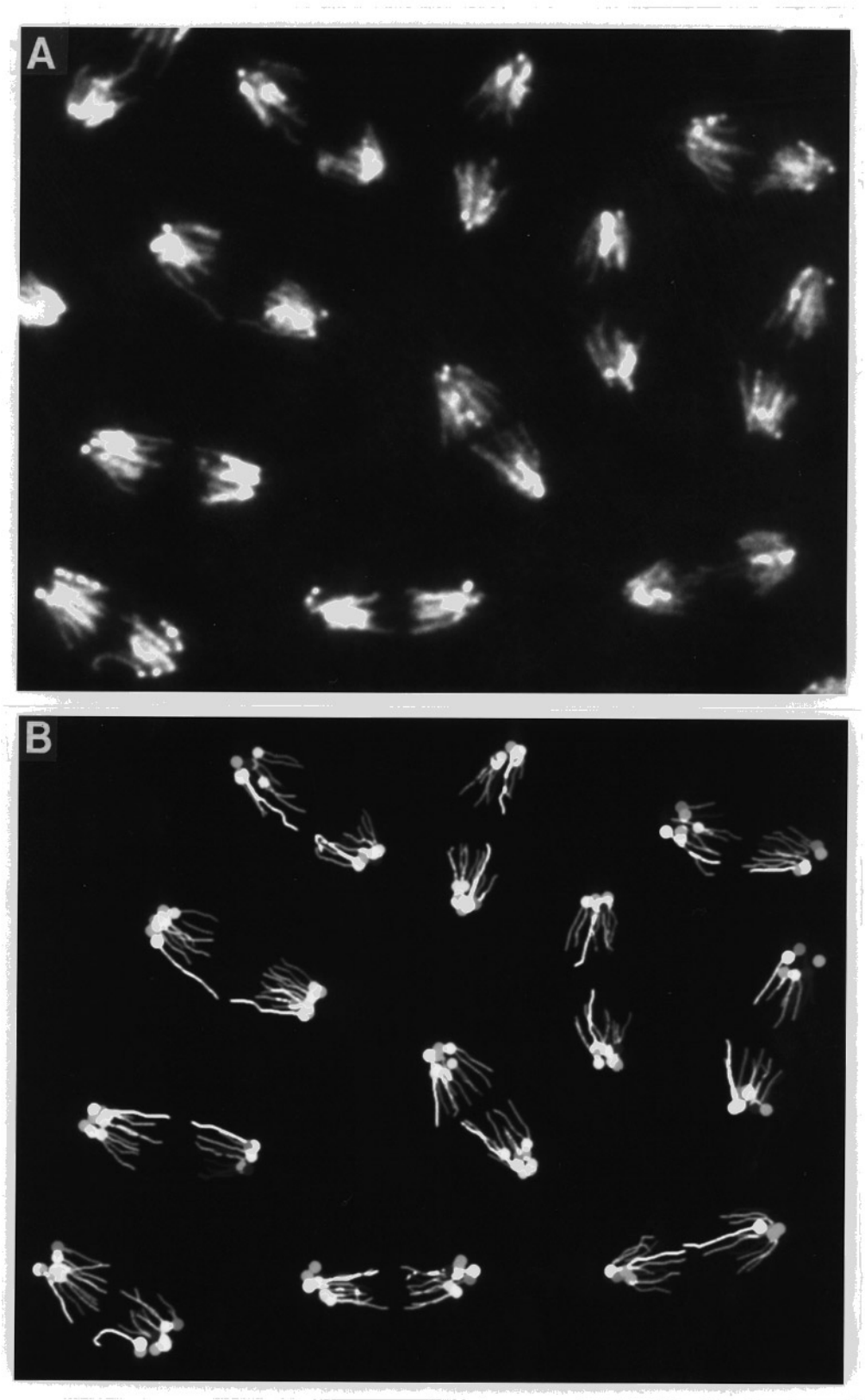

Figure 1. Path tracing of anaphase chromosomes. Anaphase chromosomes in a fixed embryo stained with DAPI (A) showing traced paths of chromosomes (B). Note that nuclei are dividing synchronously, forming a monolayer of nuclei just beneath the embryo surface.

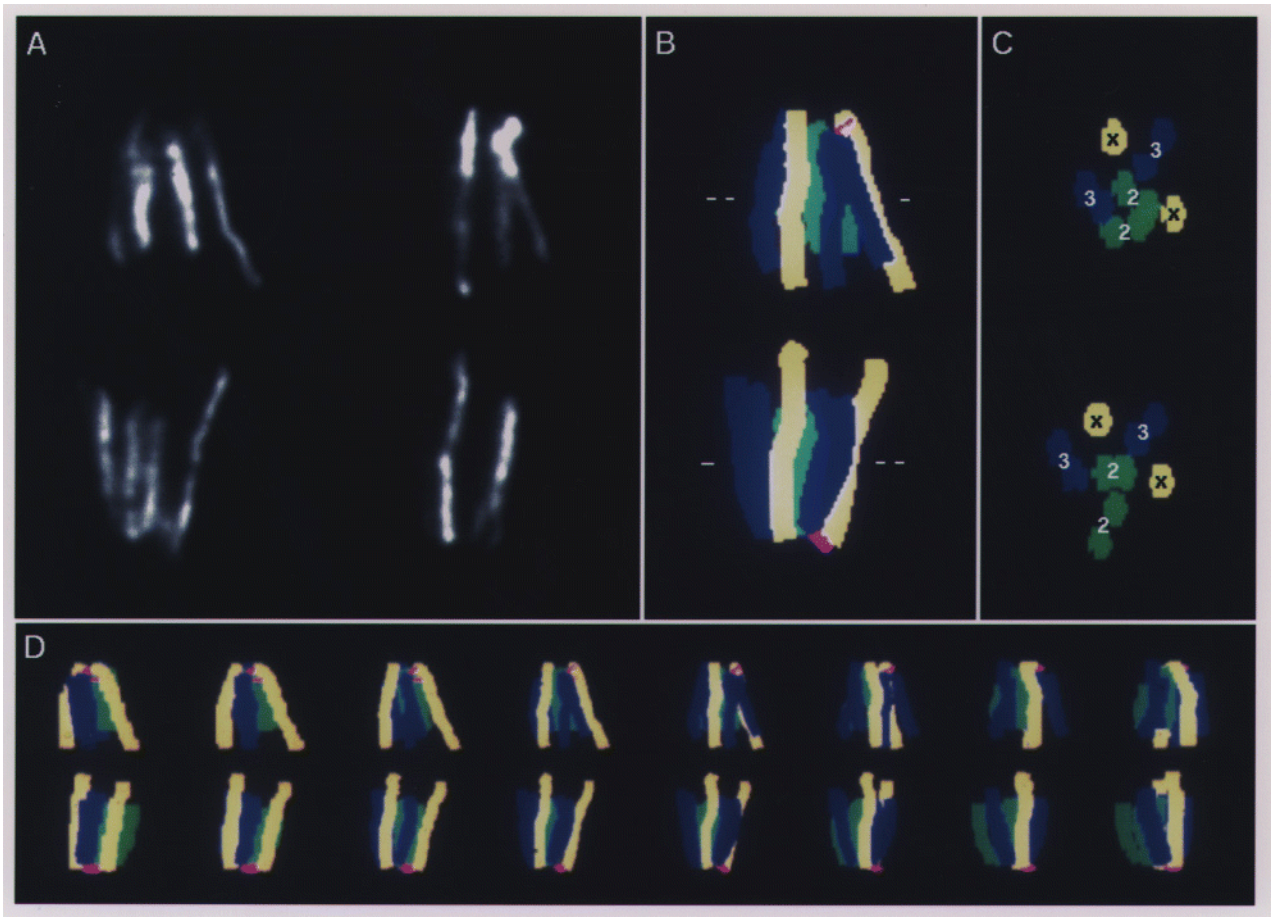

Figure 2. Chromosome arrangement in a daughter set of anaphase chromosomes. (A) Computationally-processed, optical section images of anaphase chromosomes at two focal planes separated by 2 μm. (B) Computational chromosome painting generated from chromosomes shown in (A). Color coding: yellow, X chromosome; green, chromosome 2; blue, chromosome 3; purple, chromosome 4. (C) Edge view of anaphase chromosomes at two positions for daughter sets of chromosomes indicated by white lines in B. (D) Rotated view of anaphase chromosomes, each separated by angles of 15°.

were microinjected to *Drosophila* embryos 30 min after oviposition. The injected histones were incorporated into chromosomes during periods of DNA replication, allowing visualization of chromosomes in the living state. The method is described in Minden *et al* (1989).

Embryos were illuminated with a 100 W mercury arc lamp and observed on the Olympus IMT-2 microscope using an Olympus oil-immersion objective lens (DApo 40×/NA = 1.3) and a high selectivity rhodamine filter combination (Omega Optical, Brattleboro, Vermont). The CCD pixel size corresponds to 0.17 μm in the specimen plane. In order to speed up image readout, images were taken on a small portion of the CCD, 256 × 256 pixels corresponding to a 44 × 44 μm^2 area. Five optical section images were taken by stepping the microscope focus in increments of 1 μm with an exposure time of 0.1 s. It took 5 s to read out each image and thus 25 s for each cycle of a focus series. This was repeated continuously over time.

3. Results

3.1. Arrangement of anaphase chromosomes in fixed embryos

Embryos of *Drosophila melanogaster* at the syncytial blastoderm stage were fixed with formaldehyde and stained with a DNA specific fluorescent dye, DAPI. Three-dimensional images were obtained on a cooled CCD by stepping the microscope focus at 0.2 μm intervals. Microscopic images were computationally processed to remove the out-of-focus information.

Paths of chromosomes were traced in three dimensions using an interactive computer program (see section 2). A diploid nucleus in *Drosophila melanogaster* has two sets of five chromosome arms X(Y), 2L, 2R, 3L and 3R, while chromosome four is tiny and recognized only as a dot. Thus, ten chromosome arms are expected to be visible in anaphase if they are all spatially separated from each other. Examples of anaphase chromosomes, together with traced paths of chromosomes, are shown in

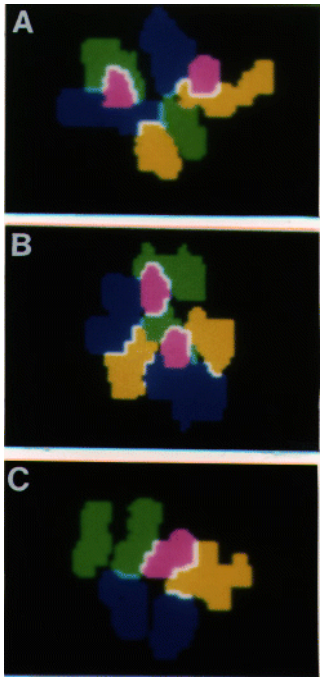

Figure 3. Projected view of anaphase chromosome domains. Anaphase chromosomes in one of the daughter sets were projected and viewed from the direction of the spindle pole. Domains occupied by each chromosome are displayed with each color. Color coding is the same as in figure 2.

figure 1. Ten chromosome arms were counted in all 33 cases from the four embryos examined, thereby indicating that homologous chromosomes are not associated with each other.

Once the chromosome paths were traced, each of the individual chromosomes in anaphase could be identified with the aid of the characteristic DAPI staining pattern for each chromosome (Hiraoka *et al* 1990a; also see figure 6). Figure 2 shows an example of the complete identification of anaphase chromosomes. Computationally-processed images are shown for two different focal planes separated by 2 μm in figure 2(A). Examples of computational chromosome painting are shown in figures 2(B)–(D). A chromosome model with pseudocolor for each of the identified chromosomes is shown in figure 2(B). Edge views of the same model are shown in figure 2(C) and the model viewed from different view angles is shown in figure 2(D). Separate sets of homologous chromosomes can be seen in figures 2(C) and 2(D).

Complete identification of chromosomes was made in a limited number of anaphase examples. All individual chromosomes were identified in 14 out of the 33 sets of anaphase chromosomes. A projected view of computational chromosome painting, viewed from the direction of the spindle pole, is shown in figure 3 for three examples. Domains occupied by chromosomes in an anaphase figure are each represented by a color. Because the relative positions of individual chromosomes in each of the daughter sets of anaphase chromosomes were mirror symmetrical to each other (as can be seen in figure 2), only one of the daughter sets is displayed as a representative in figures 3 and 4. Anaphase chromosome arrangement for 14 examples is shown in figure 4. Pairs of homologous chromosomes were not necessarily in close apposition but were often interrupted by heterologous chromosomes.

3.2. Arrangement of anaphase chromosomes in living embryos

The separate location of homologous chromosomes observed in fixed embryos was augmented by analysis of living embryos. In living embryos, unlike in fixed embryos, sufficient microscopic resolution to identify each of the individual chromosomes could not be obtained due to current technological limitations (see section 4). To compensate for the lower resolution obtainable in living embryos, we used the ltx13 strain, which bears a significantly long chromosome arm. In the ltx13 strain, almost the entire arm of the chromosome 2L is translocated to the end of chromosome 3R (Wakimoto and Hearn 1990). The translocated chromosome arm 3R/2L can be distinguished from the rest of the chromosome arms by length, as the lengths of chromosome arms in the wild type strain are not significantly different from each other.

Thus we examined the arrangement of homologous chromosomes in the homozygous ltx13/ltx13 strain. Chromosomes in living embryos were stained by microinjection of histone proteins conjugated with a fluorescent dye, rhodamine (Minden *et al* 1989, Hiraoka *et al* 1989). Figure 5 shows four sets of anaphase chromosomes in living embryos of the homozygous ltx13/ltx13 strain. Two long arms can be seen in all four examples (indicated by arrows in two examples), showing that a homologous pair of the translocated chromosomes are spatially separated from each other in anaphase.

3.3. Arrangement of chromosomes in anoxic embryos

Embryos kept in anoxic conditions show a characteristic swollen nuclear morphology (Foe and Alberts 1985). The diameter of nuclei in anoxic embryos is about two times that of a normal nucleus. In anoxic prophase nuclei, condensed chromosomes are located primarily immediately beneath the swollen nuclear envelope, while centromeres are localized in a nuclear region near the embryo surface and telomeres are in the other side of the nucleus toward the embryo interior (Foe and Alberts 1985). This polarized centromere–telomere configuration with centromeres pointing toward the embryo surface is a characteristic feature of chromosome arrangement in normal *Drosophila* embryos at the syncytial blastoderm stage, as well as in anoxic embryos (Hiraoka *et al* 1990a). In such nuclei under anoxic conditions, individual chromosomes were sparsely located within a spacious

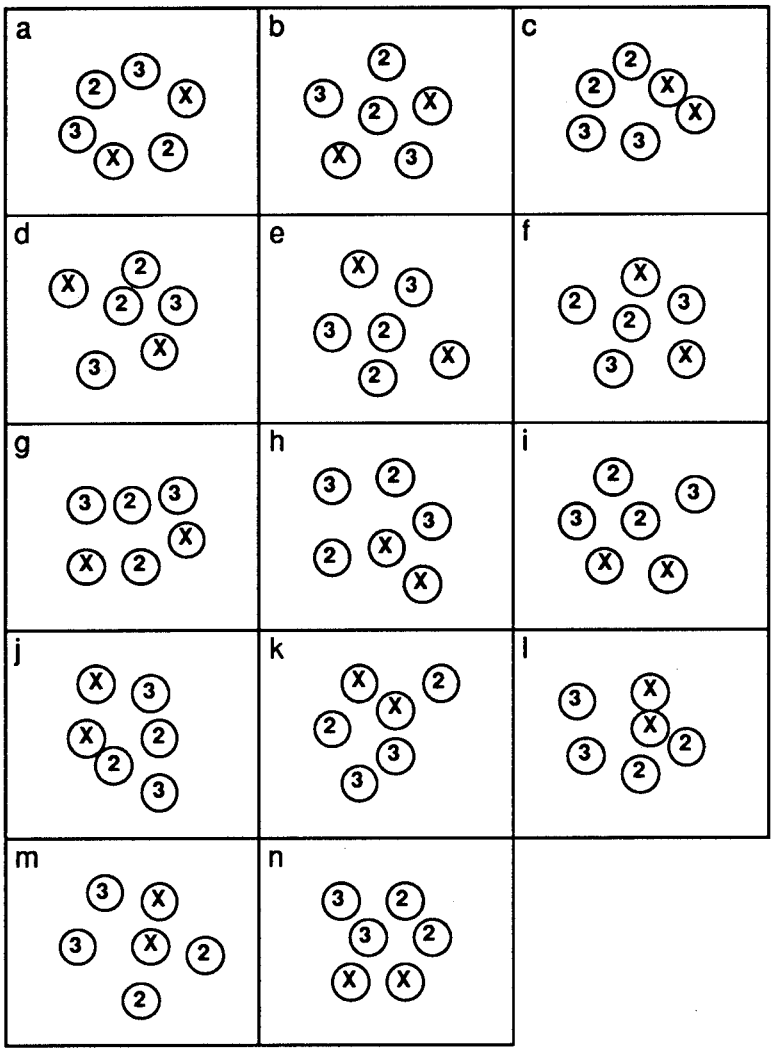

Figure 4. Arrangement of anaphase chromosome domains. The position of each identified chromosome is shown for 14 examples.

volume of a swollen nucleus and chromosomes could be identified by the DAPI staining pattern of pericentric regions. Two sets of all chromosomes were distinguished by visual inspection as displayed by computational chromosome painting in figure 6, indicating that a pair of homologous chromosomes have no physical association with each other.

3.4. Compaction of DNA in chromosomes

Chromosomes condense at prophase, reach the maximum level of condensation at metaphase, and decondense after chromosome segregation at telophase. Tracing the path of chromosomes in three dimensions allowed us to measure the length of individual chromosomes. Total lengths of diploid chromosomes were 34.32 ± 1.21 μm in anaphase (28 samples) and 101.9 ± 13.8 μm in anoxic prophase (eight samples). The compaction ratio of DNA in chromosomes can be calculated from the total length of chromosomes by comparing with the amount of DNA. The haploid genome of *Drosophila melanogaster* consists of 160 Mbp corresponding to 55 mm of naked DNA. Therefore, the compaction ratio of DNA was about 3000 at anaphase and 1100 in anoxic prophase. Calculated from the previous study, the compaction ratio is 1600 at prophase (Hiraoka *et al* 1990a). Assuming that anaphase chromosomes retain a similar level of condensation to metaphase chromosomes, metaphase/anaphase chromosomes condense $\sim$2–3 times from prophase chromosomes. These values were compared with those for models of higher-order chromatin structures. The compaction ratio of DNA in nucleosomes, solenoids and supersolenoids is estimated as 7, 42–80 and 800–1600, respectively, with variations depending on the models; the final level of compaction varies according to species and the compaction ratio is 5000–8000 in human metaphase chromosomes (reviewed in Sedat and Manuelidis 1978).

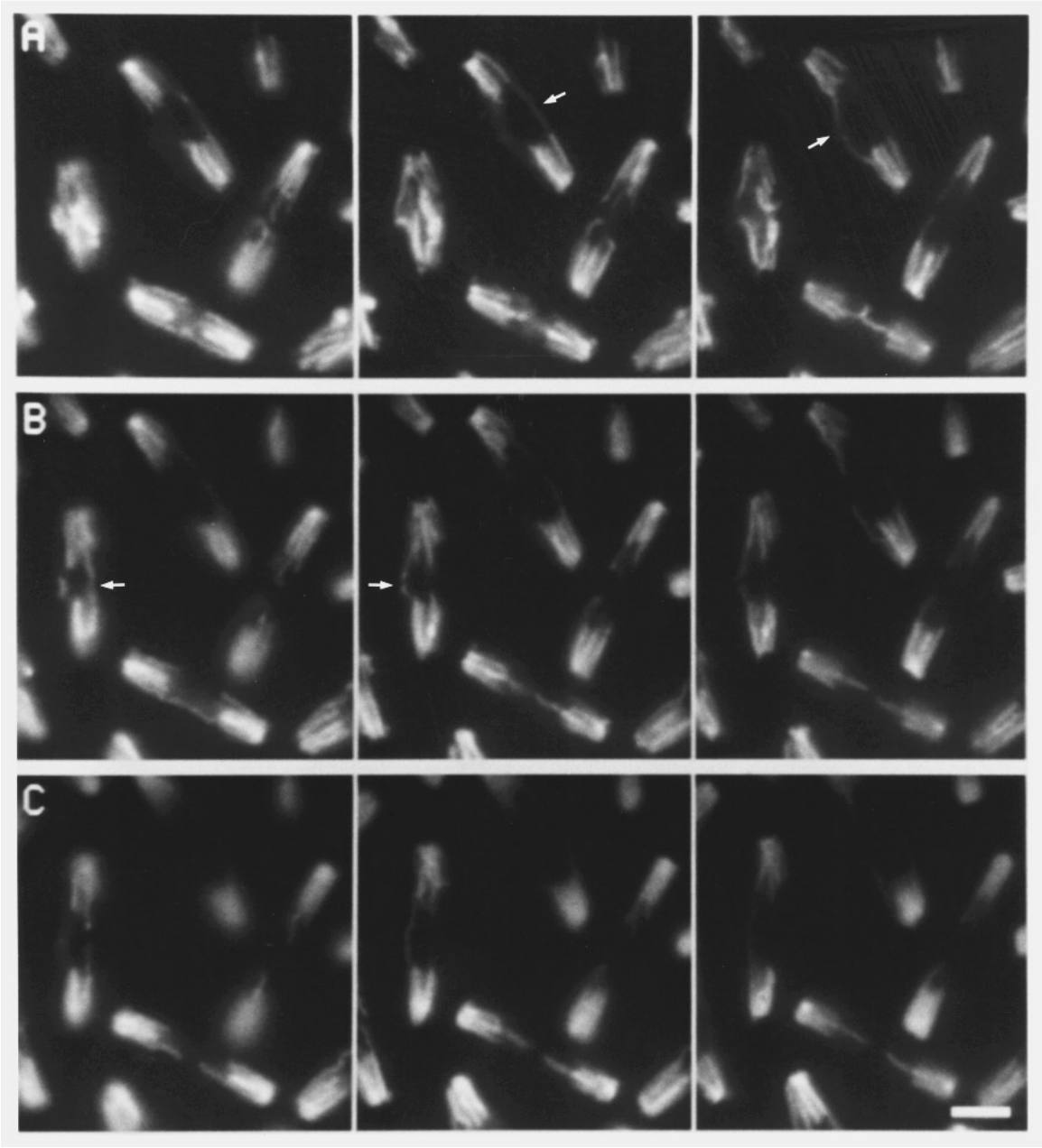

Figure 5. Anaphase chromosome dynamics in a living embryo of the *Drosophila* ltx13 strain. The focus series from the embryo interior to the surface at 1 μm intervals are displayed from left to right. Optical section data are shown for three time points A, B and C obtained every 25 s. The long translocated chromosome arm is indicated by an arrow. The bar indicates 5 μm.

Thus, the compaction ratios of *Drosophila* chromosomes fits in these models with a variation in the final level of metaphase chromosomes; the final level of compaction is about two times less condensed in *Drosophila* than in man.

4. Discussion

We have examined the spatial arrangement of homologous chromosomes during anaphase in fixed and living embryos of *Drosophila melanogaster* at the syncytial blastoderm stage. In fixed embryos, high-resolution three-dimensional images can be obtained by computational image processing and the paths of the chromosomes within a nucleus can be traced in three dimensions. For computational chromosome painting, the chromosomes are identified, dissected and painted with pseudocolor as previously described (Chen *et al* 1989, 1996, Hiraoka *et al* 1990a). High-resolution analysis of anaphase chromosomes in fixed embryos indicated that homologous chromosomes were not associated with each other.

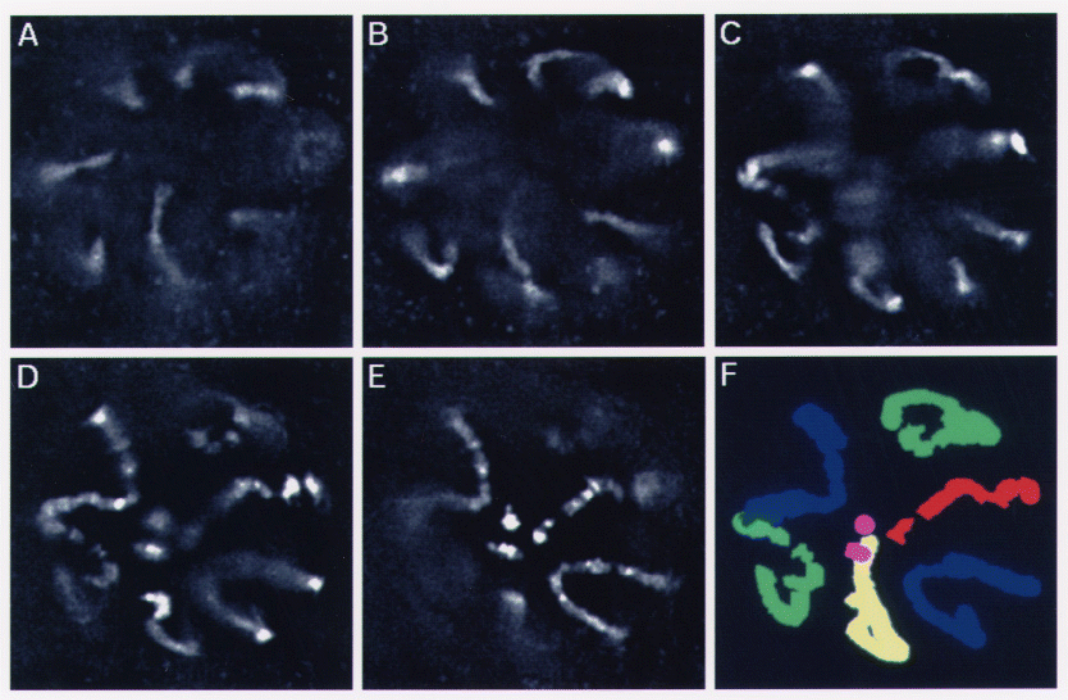

Figure 6. Chromosome arrangement in anoxic prophase. (A–E) Computationally-processed, optical section images of anoxic prophase chromosomes at focus intervals of 0.5 μm. (F) Computational chromosome painting generated from chromosomes shown in (A–E). Color coding: yellow, X chromosome; red, Y chromosome; green, chromosome 2; blue, chromosome 3; purple, chromosome 4.

An ideal complement to high-resolution analysis in fixed embryos is continuous observation in living embryos, which provides a unique opportunity to examine the behavior of chromosomes over time. In living embryos, however, sufficient microscopic resolution to identify each of the individual chromosomes cannot be obtained. In the case of fixed specimens in which a large number of optical sections with fine focus intervals to generate high-resolution three-dimensional images can be obtained. However, with living embryos only a limited number of optical sections can be collected. This is necessary in order to keep up with the dynamic processes of living specimens, and also to avoid the phototoxicity to fluorescently-stained living cells. In practice, we recorded a three-dimensional data stack with typically 40 to 50 optical sections at 0.2–0.25 μm focus intervals in fixed embryos, whereas only five images at 1 μm focus intervals were obtained at each point in time in living embryos. Thus, technological improvement in the speed of image acquisition and the sensitivity of image detectors is important in order to further improve available resolution in living specimens. In this report, we used a strain bearing chromosomal translocation to compensate for the current technological limitations in imaging living embryos. Because a large number of strains carrying chromosome aberrations such as translocation and inversion have been obtained in *Drosophila* (Lindsley and Zimm 1992), the use of such strains greatly facilitates the analysis of chromosome arrangement and dynamics. Our observation of the translocated long chromosome in living embryos confirmed the separate location of homologous chromosomes during anaphase as obtained from fixed embryos, and eliminated the possibility of artifacts in preparation of fixed specimens.

Another cytological tool that we used was the maintenance of an artificial level of chromosome condensation during early prophase under anoxic conditions. Anoxic prophase chromosomes in fixed embryos indicated that homologous chromosomes were separated from each other. The fact that individual chromosomes remain separate during the process of chromosome condensation under anoxia suggests that chromosomes are not intermingled with each other within a nucleus. Separate domains of chromosomes in an interphase nucleus have been observed in a wide range of animals and plants (Manuelidis 1985, van Dekken *et al* 1989, Heslop-Harrison and Bennett 1990, Cremer *et al* 1996).

Previous studies have shown that homologous chromosomes are separated from each other in prophase and metaphase (Hiraoka *et al* 1990a). In addition, homologous loci of histone genes are separated from each other in interphase nuclei (Hiraoka *et al* 1993). Combining these results, we concluded that homologous chromosomes are not associated with each other throughout the cell cycle at this early developmental stage. We wish to emphasize, however, that this conclusion does not eliminate the possibility that somatic pairing of homologous chromosomes takes place at later developmental stages, as inferred by genetic evidence of transvection effects. If homologous

chromosomes are paired at later stages, rearrangement of chromosomes to bring a homologous pair in close apposition must take place during the process of embryogenesis. In fact, analysis by *in situ* hybridization has shown that the homologous loci of histone genes initially become paired at the 14th nuclear division (Hiraoka *et al* 1993), and that homologous pairing proceeds further at other chromosomal loci along the chromosome at later stages, ultimately leading to fully paired homologous chromosomes in somatic cells (Fung *et al* 1997).

We have described the results of our study of chromosome arrangement and dynamics obtained by combining imaging technology with cytological tools such as the use of translocated chromosomes *in vivo*, and the use of artificially condensed chromosomes under anoxia. Such cytological tools can greatly facilitate the dissection of nuclear structures by complementing the imaging technology.

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