Three-dimensional organization of chromosomes studied by

*in situ* hybridization and optical sectioning microscopy

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ABSTRACT

We have examined the three-dimensional arrangement of chromosomes in embryos of *Drosophila melanogaster* at the syncytial blastoderm stage using three-dimensional optical sectioning microscopy. High-resolution optical sectioning in fixed embryos, in conjunction with *in situ* hybridization, has revealed the location of specific chromosomal regions within diploid interphase nuclei, as well as the spatial arrangement of mitotic chromosomes. Time-lapse *in vivo* optical sectioning has revealed the dynamic behavior of chromosomes throughout the mitotic cycle. Combination of these observations has provided insights into the dynamic aspects of three-dimensional chromosome behavior.

2. INTRODUCTION

Chromosomes are dynamic and ephemeral structures, changing their structure and organization throughout the cell cycle. Until recently, it has only been possible to visualize chromosomes in light microscopy at certain times in the diploid cell cycle when chromosomes are condensed, namely prophase through telophase, or in certain specialized tissues containing polytenized giant chromosomes. In order to obtain a more complete understanding of the structure and behavior of diploid chromosomes throughout the entire cell cycle, we have examined the three-dimensional arrangement of chromosomes in early embryos of *Drosophila melanogaster*. In early *Drosophila* embryos, up to 5000 nuclei divide rapidly and essentially synchronously every 10-20 min; providing an attractive biological system for studying mitotic processes.

Our analyses were accomplished by three-dimensional optical sectioning microscopy techniques, using a cooled scientific-grade charge-coupled device (CCD) to record the microscopic data (Hiraoka et al., 1987; Aikens et al., 1989) and computational image processing to remove the out-of-focus information (Agard et al., 1989). The dimensional and photometric accuracy of CCD image data allowed computational image processing to improve the microscopic resolution for study of the spatial arrangement of mitotic chromosomes in embryonic diploid nuclei. Because these chromosomes are so small (0.2-0.4 μm width) and packed into a small nucleus (4-5 μm diameter), image processing is absolutely necessary.

In an effort to follow mitotic processes through the entire cell cycle, we have extended our three-dimensional imaging technologies in two new directions: *in situ* hybridization and *in vivo* optical sectioning. *In situ* hybridization is a technique that labels specific chromosomal regions by localizing fluorescently -tagged DNA probes complementary to chromosomal nucleotide sequences (Pinkel et al., 1986, 1988; Lawrence et al., 1988; Rykowski et al., 1988). High-resolution optical sectioning, in conjunction with *in situ* hybridization, has revealed the three-dimensional location of specific genetic loci within diploid interphase nuclei. Furthermore, it is now possible to examine living samples in three dimensions by time-lapse, optical sectioning microscopy (Minden et al., 1989; Hiraoka et al., 1989). This is an ideal complement to high-resolution analysis in fixed preparations, providing the unique capability of continuous observation of individual nuclei over time. Here we describe the three-dimensional microscopy techniques for studying the spatial arrangement and dynamic behavior of diploid chromosomes during the entire cell cycle.

3. THREE-DIMENSIONAL FLUORESCENCE MICROSCOPY

3.1. Data collection

Three-dimensional data are collected as a series of digital images recorded at different focal planes within the specimen by stepping a microscope focus under computer control. We have developed two schemes
for data collection based on optical sectioning, each with unique advantages: a time-lapse optical sectioning in living embryos and a high-resolution optical sectioning in fixed embryos. The high-resolution scheme allows the determination of paths of mitotic chromosomes with cytological identification. When combined with in conjunction with in situ hybridization, it is possible to localize specific chromosomal sequences within diploid interphase nuclei. The time-lapse recording scheme provides a unique opportunity to analyze the dynamic behavior of chromosomes in individual nuclei as a function of time.

For recording and analyzing three-dimensional image data on a routine basis, a standalone computer workstation has been developed. All aspects of microscopy, data collection, data storage, image processing and display can be performed on the one workstation. A pellicier-cooled CCD camera (Photometrics, Tucson, Arizona) with a 1340x1037 pixel CCD chip (Kodak-Videk, coated to improve the short-wavelength response) is attached to an Olympus IMT-2 inverted microscope via the regular camera port. For rapid wavelength-switching during data collection, excitation filters and barrier filters are mounted on revolving wheels, making it possible to record images of multiple macromolecular components that are differentially stained with fluorescent dyes. A dichroic mirror that has double- or triple-wavelength band-pass properties (Omega Optical, Inc. Vermont) is used in the multiple-label experiments to eliminate the displacement of images. The microscope focusing motor, lamp and CCD shutters, wavelength switching, and CCD data collection are controlled by a MicroVax III workstation coupled to a 20 MLOPS Mercury Zip 3232+ array processor (Dermatologic Systems Inc., Lowell, Mass.) and a Parallax model 1280 graphic display system having 12 Mbyte of image memory (Parallax Graphics, Inc., Santa Clara, California).

3.2. Removal of out-of-focus information

Image-forming properties of a microscope are best characterized by the point-spread function (PSF) which describes how an idealized point source is spread by the passage through the microscope. A microscopic image is then assumed to be the result of a convolution of the "true" image with the PSF:

$$i(x,y,z) = \int \int \int o(x',y',z') \cdot PSF(x-x',y-y',z-z') \, dx'dy'dz'$$

where i is the observed image, o the object or "true" image, and the z index refers to the focus direction. Recasting equation 1 in terms of its Fourier transform and recalling the optical transfer function (OTF) is the Fourier transform of the PSF:

$$I(u,v,w) = O(u,v,w) \cdot OTF(u,v,w)$$

where capital letters refer to the Fourier transforms of the corresponding lower-case quantities, (u,v,w) are the Fourier space analogs of (x,y,z), and the convolution is now simplified to a multiplication.

Three-dimensional image stacks are computationally processed to remove out-of-focus image information by a constrained iterative deconvolution method using the three-dimensional OTF for the objective lens used (Agard, 1984; Agard et al., 1989). For this purpose, the PSF was experimentally determined under the conditions used for the data collection of nuclei by imaging very small fluorescent beads (0.1μm in diameter); the three-dimensional OTF was calculated by taking the Fourier transformation of the PSF (Hiraoka et al., 1990; Hiraoka et al., 1987).

Alternatively, when only stereo pairs of projected images, instead of individual section images, are needed, images can be first projected and then processed to remove out-of-focus information by two-dimensional deconvolution using the w=0 plane of the OTF (Agard et al., 1989). In practice, OTF(u,v,l) is determined by taking the Fourier transformation of the projected PSF. This is a simple and straightforward process, based on the Fourier projection theorem, that can provide nearly exact deblurring. Furthermore, since no light is rejected in the image processing, this approach is ideal for low-light level imaging as is required for in vivo analysis (see Fig. 3 below; Hiraoka et al., 1989).

It should be noted that real observation conditions for biological specimens are often far from ideal for three-dimensional imaging. In order to remove the out-of-focus information effectively, it is important to characterize the optical behavior of the microscope system under the observation conditions being used, and to optimize the observation conditions if necessary. Especially important is minimizing the residual spherical aberration. If this is not taken into account, the resultant images can be seriously degraded. For detailed discussion, see Hiraoka et al. (1990).
3.3 Image analysis

An important part of our approach is to provide a framework for the analysis and interpretation of the reconstructed data. Typically, grey level images are first processed to remove the out-of-focus information, and then further processed, as necessary, to enhance local contrast (Belmont et al., 1987), to detect chromosome boundaries using three-dimensional intensity gradient (see Fig. 2 below), or to generate a set of projected stereo images corresponding to rotated views (Agard et al., 1989, Chen et al., 1989). Here we specifically describe methods for the analysis of the spatial arrangement of chromosomes within a nucleus.

Chromosome paths are traced in three dimensions using an interactive computer program PRISM (Chen et al., 1989). This program can open up to 8 windows on a display screen at one time and display computationally-rotated multiple views of the same object in each window. Chromosome paths are traced by a cursor and recorded as sets of three-dimensional coordinates, while the multiple images from various view angles in other windows are updated simultaneously to follow the cursor movement. Modelling and image display are carried out on the Parallax graphic display.

Once chromosome paths are determined, a computational dissection scheme can be used to facilitate visualization of structural features of complicated chromosome paths. Grey level information associated with the selected chromosome paths is discriminated by thresholding the grey level intensity and/or detecting chromosome edges based on the intensity gradient, and dissected from the rest of chromosomes. The dissected chromosomes are recomposed as the outlines filled with a particular color (see Fig. 1 below). In addition, the dissected chromosomes can be computationally straightened by a combination of operations of rotation, translation and transformation based on three-dimensional geometry. These capabilities greatly facilitate the interpretation of complex images.

4. THREE-DIMENSIONAL CHROMOSOME ORGANIZATION

4.1. Spatial arrangement of mitotic chromosomes

We wished to determine three-dimensional paths of chromosomes within diploid nuclei at various cell cycle times, beginning at prophase when chromosomes first become visible, continuing through metaphase, and to telophase before they totally decondense. Embryos of Drosophila melanogaster were fixed with 3.7% formaldehyde under conditions previously shown to preserve chromosome structures in electron microscopy (Belmont et al., 1989) and stained with the non-intercalative DNA-specific fluorescent dye, 4',6-diamidino-2-phenylindole (DAPI). Optical section images were collected on the CCD at focus steps of 0.2μm or 0.25μm using high numerical aperture objective lenses (NA 1.2-1.4). Out-of-focus information was removed to improve the microscopic resolution. Chromosome paths were traced in three dimensions using the interactive modelling program. Computational "chromosome painting" was made by computationally dissecting chromosomes and recomposing them filled with a pseudocolor. Fig. 1 shows optical section images of prophase chromosomes at different focal planes (A), together with pseudocolor presentations of the chromosomes (B). A stereo view made from the pseudocolor chromosomes is presented in Fig. 1C.

This approach has revealed the spatial arrangement of chromosomes from prophase to telophase. Our results shows that the chromosomes in embryonic diploid nuclei are arranged, very like those in polytene nuclei, in a polarized orientation from centromeres to telomerces, and that this polarized arrangement is maintained in an unexpected degree of ordering during mitosis. However, unlike polytene nuclei, homologous chromosomes are not associated at prophase through telophase.

4.2. Organization of interphase chromosomes

In interphase when the essential processes of gene transcription and DNA replication occur, chromosomes are too decondensed to be traced using current approaches, so we wish to use specific labelling schemes to extract salient features. One specific biological question is whether homologous chromosomes are associated in interphase. The three-dimensional structure of the diploid nucleus can be dissected using high-resolution in situ hybridization to whole-mount embryos. In this procedure, biotinylated probes homologous to chromosomal sequences are hybridized in situ to embryos that were previously fixed to preserve chromosome structure. The location of the hybridization probes is revealed by staining these embryos with fluorescently-
tagged avidin molecules. Optical sectioning of such embryos reveals the three-dimensional location of the DNA sequence relative to chromosomal structures within the nucleus. Other schemes of detection are also available. Combination of multiple detection schemes using different fluorescent dyes can be used for differential staining of chromosomal regions (Cremer et al., 1988; Hopman et al., 1988).

Fig. 2 shows an example of in situ hybridization to embryos. Biotinylated DNA probes homologous to 8-20 kb single-copy chromosomal sequences were hybridized in situ to embryos and detected with fluorescently-labelled avidin molecules. The same DNA probes were hybridized to polytene chromosomes. The DNA sequences are located near the end of the X chromosome. The results are shown in Fig. 2 in the same magnification for polytene (B) and embryonic prophase chromosomes (D). Optical sectioning of these embryos revealed that the DNA sequences are localized at the bottom of the nucleus. Another example of in situ hybridization to embryos is shown in Fig. 2G. In this example, DNA probes homologous to a 200 kb single-copy sequence that is located at the middle of the chromosome arm 2R was hybridized to embryonic interphase nuclei. Optical sectioning revealed that this DNA sequence is localized at the midline of the nuclei. These results of hybridization are consistent with the ordered arrangement of interphase chromosomes in embryonic nuclei with centromeres near the embryo surface and telomeres diametrically opposed as shown in prematurely condensed chromosomes (Foe and Alberts, 1985).

We are currently using a wide range of DNA probes for specific chromosomal sequences to make the "chromosome painting" in interphase nuclei by molecular dissection. Our preliminary results using DNA probes to autosomal chromosomes suggest that homologous chromosomes are not associated in interphase at least at this developmental stage of embryos. This apparently contradicts genetic experiments of transvection which suggest the homologous chromosomes are associated (Lewis, 1954; Green, 1959; Jack and Judd, 1979; Gelbart, 1982).

4.3. Chromosome dynamics in living embryos

In order to follow the dynamic behavior of chromosomes during the entire cell cycle, continuous observation in living specimens is necessary. This was accomplished by three-dimensional, time-lapse microscopy in conjunction with the stereographic projection and computational processing protocols (Fig. 3). Chromosomes were visualized by microinjection of rhodamine-labelled calf thymus histones H2A and H2B to living embryos (Minden et al., 1989). The projected stereo images were computationally processed to remove out-of-focus information by a constrained iterative deconvolution method using the OTF(u,v,0) as described above (Hiraoka et al., 1989). Because of a compromise between temporal and spatial resolution, data need to be collected at lower spatial resolution in living specimens than in fixed specimens. However, combined with high-resolution analyses, this approach uniquely provides the dynamic aspects for the structures seen in the fixed preparations.

This approach was particularly useful for studying the dynamics of chromosome condensation and decondensation between telophase and next prophase. Our results have revealed that chromosomal regions on the nuclear envelope, distinct from the centromeres and telomeres, serve as foci for the decondensation and condensation of diploid chromosomes (Hiraoka et al., 1989).

5. DISCUSSION

Since the first demonstration of the polarized arrangement of chromosomes with centromeres and telomeres on each end of the nucleus (Rabl, 1885), ordered arrangements of chromosomes in interphase nuclei have been inferred in a wide range of species (reviewed in Avivi and Feldman, 1980; Comming, 1980; Fussel, 1984). However, it has not been possible to directly follow the paths of chromosomes throughout the cell cycle. Thus, in order to study the dynamic aspects of three-dimensional nuclear structures, we have examined diploid nuclei in Drosophila embryos using several complementary schemes of three-dimensional optical sectioning microscopy. The combination of these approaches has provided us new insights into complex and dynamic behavior of chromosomes.
6. ACKNOWLEDGEMENTS

We thank Susan Parmelee for preparation of and hybridization to polytene chromosomes. This work was supported by grants from National Institute of Health to J. W. Sedat (GM-25101) and D. A. Agard (GM-31627). J. W. Sedat and D. A. Agard are Howard Hughes Investigators. D. A. Agard is also a National Science Foundation Presidential Young Investigator. Y. Hiraoka was supported by Damon Runyon - Walter Winchell Cancer Research Fund Fellowship DRG903. M. C. Rykowski's present address is University of Arizona, Department of Anatomy, Tucson, Arizona 95720.

7. REFERENCES


FIGURES

Fig. 1  Chromosome painting by high-resolution optical sectioning and computational dissection.  
(A) Optical sections from a three-dimensional data stack after the removal of out-of-focus image 
contamination.  Three-dimensional image data were obtained at a 0.25μm focus interval under 
computer control.  Each displayed section is separated by 0.5μm in z. 
(B) Pseudocolor presentation of chromosomes (pseudocolors are displayed as grey levels in this 
figure). Each of chromosomes shown in A was traced and identified. Chromosomes were 
computationally dissected by isolating cylinders surrounding the the traced paths and 
thresholding the grey level intensity.  
(C) Recomposed from the dissected individual chromosomes with pseudocolor for each 
chromosome (pseudocolors are displayed as grey levels as in B). Displayed as a stereo pair. 
Scale bar represents 1 μm.

Fig. 2  Localization of specific chromosomal sequences by in situ hybridization.  
(A,B) A squash preparation of Drosophila polytene chromosomes. A portion near the end of X 
chromosome with DAPI-staining (A) and in situ hybridization (B).  
(C-E) A section of three-dimensional data of embryonic nuclei, at the 13th prophase, stained 
with DAPI (C) and in situ hybridization (D). In E, sites of hybridization signals are 
superimposed on the DAPI-stained chromosomes displayed as the intensity gradient. DNA 
probes used in B and D are a mixture of three sequences for zeste (17 kb), white (11 kb) and Notch 
(30 kb) genes in the order from the end of the chromosome.  
(F-H) A section of three-dimensional data of embryonic nuclei, at the 14th interphase, stained 
with DAPI (F) and in situ hybridization(G). In H, sites of hybridization signals are 
superimposed on the outline of nuclei displayed as the intensity gradient. DNA probes span about 
200 kb around the engrailed gene.

Fig. 3  Three-dimensional in vivo microscopy.  
(A) Recording three-dimensional data from living embryos. Five optical sections of a through-
focus series at 1μm intervals are shown as a function of time.  (B) Stereo pairs selected from the 
time-lapse data. To make a pair of stereo projections, each set of 5 optical sections was projected 
while sequentially shifting images for each eye. The projected stereo images were 
computationally processed to remove the out-of-focus information. The figure was reproduced 
from Hiraoka et al. (1989).
Fig. 1 Chromosome painting by high-resolution optical sectioning and computational dissection.
Fig. 2 Localization of specific chromosomal sequences by *in situ* hybridization.
Fig. 3 Three-dimensional in vivo microscopy.