

Three-Dimensional Light Microscopy of Diploid *Drosophila* Chromosomes

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Fluorescence microscopy, uniquely, provides the ability to examine specific components within intact, even living, cells. Unfortunately, high-resolution conventional fluorescence microscopy is intrinsically a two-dimensional technique and performs poorly with specimens thicker than about 0.5 μm . Probing the spatial organization of components within cells has required the development of new methods optimized for three-dimensional data collection, processing, display, and interpretation. Our interest in understanding the relationship between chromosome structure and function has led us to develop the necessary methodology for exploring cell structures in three dimensions. It is now possible to determine directly the three-dimensional spatial organization of diploid chromosomes within intact nuclei throughout most of the mitotic the cell cycle.

Key words: chromosome structure, spatial organization, optical sectioning

INTRODUCTION

In an effort to understand the complex relationship between structure and biological function within the nucleus, we have embarked on a program to examine the three-dimensional structure and organization of *Drosophila melanogaster* chromosomes. Our goal is to determine how DNA and proteins, as chromosomes, are organized into complex and highly dynamic structures and how these entities are arranged in space within the nucleus. Furthermore, we hope to be able to correlate structural data with such fundamental biological properties as stage in the mitotic cell cycle, developmental state, and transcription at specific gene loci.

Towards this end, we have been developing methodologies for the three-dimensional analysis of noncrystalline biological specimens using light and electron microscopy. We believe that the combination of these two complementary techniques allows an unprecedented look at structural organization of cellular components ranging in size from 100 \AA to 100 μm . Light microscopy, uniquely, allows very large biological structures to be examined in situ under living or nearly native conditions. Fluorescence microscopy combined with optical sectioning methodologies [for review, see Agard, 1984] allows specific macromolecular components to be visu-

alized within intact tissues in three dimensions. With modern image-processing methods, it is now possible to generate three-dimensional optical images having a resolution of about 1,500 \AA . Extending the structural analysis to sufficient resolution to understand the architecture of complex specimens at the molecular level can only be accomplished using electron microscopy. However, it is necessary to sacrifice the specificity and aqueous environment found with light microscopic methods. Conventional electron microscopy is intrinsically a two-dimensional technique optimized for very thin specimens; the size and complexity of supramolecular assemblies or cell components require that a three-dimensional analysis be pursued. By combining electron microscopic tomography with intermediate- or high-voltage electron microscopy it is possible to analyze structures in either semithick plastic sections or whole mounts, in three dimensions, at resolutions approaching 50 \AA [Belmont et al., 1987].

Because of the emphasis on light microscopy at the

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Allen Symposium, we present here only some of our initial efforts on examining the spatial arrangement of diploid chromosomes from *D. melanogaster* early embryos and the computational and optical methods that made them possible. These fluorescence microscopy studies are conceptually related to our earlier work on *Drosophila* polytene chromosomes [Agard and Sedat, 1983; Mathog et al., 1984; Gruenbaum et al., 1984; Hochstrasser et al., 1986], yet the much smaller size of diploid chromosomes (0.2–0.4 μm diameter vs. 3 μm) requires approximately a ten fold increase in resolution. This is made possible by combining state-of-the-art imaging technology [charge-coupled device (CCD) imager] with a precise knowledge of the optical behavior of the microscope.

It should be pointed out that these methodologies are entirely general ones, lending themselves to the analysis of microtubule behavior as well as chromosomal organization. Furthermore, because of significant advances in the experimental analysis of exactly how light microscopy generates (and distorts) three-dimensional images as well as in the image processing methods required to overcome these distortions, it is now possible to obtain exceptionally high quality three-dimensional optical reconstructions without recourse to giant, expensive computers. Much of the requisite image processing can be recast as convolutions and run on small, inexpensive video rate image-processing equipment of the kind often used for video-enhanced Nomarski imaging.

In our view, three-dimensional cell structure analysis has three major components: 1) data collection, 2) image processing, and 3) display and analysis. All three of these steps have required significant effort and development time. However, the effort has been justified, because it is the interrelationship of all three that ultimately leads to improved understanding of the fundamental biological issues. For light microscopy, data collection involves optically sectioning the specimen—recording of a set of images at different planes of focus. Such image recording can be done either on a conventional microscope equipped with a cooled CCD imager or on a confocal microscope. In either case, precise control over the focal plane is required, as is the recording of images free from geometric and photometric distortions.

The choice between microscopic approaches (confocal or CCD imager) will ultimately depend on such issues as photobleaching, time resolution, choice of available excitation wavelengths, existing equipment, etc. As discussed below, the nature of three-dimensional imaging is such that the generation of stereoscopic images or fully in-focus synthetic projections can be accomplished with considerably less photobleaching using conventional optical sectioning microscopy rather

than confocal methods. This may be critically important in the analysis of living cells. Using either approach, it is now possible to analyze cellular architecture of intact specimens in three dimensions.

For conventional optical sectioning microscopy, image processing is required to remove the out-of-focus information that contaminates each image. Although confocal microscopy is much better at rejecting information from adjacent image planes, it is certainly not perfect. Indeed, by image processing it should be as easy to improve dramatically resolution from confocal images as from conventional optical sections.

Once a three-dimensional set of data has been collected, it is necessary to display the information in a manner that can be readily interpreted. In our opinion, simply scanning through the sections is very inappropriate. A far more natural means for viewing three-dimensional images is via stereo pairs or, better, through sets of projected images calculated at different tilt angles. When rapidly played in succession, these create the effect of a rotating three-dimensional object. Naturally, the combination of rotating images with stereo pairs is, by far, the most informative.

For the sort of studies that we are engaged in, interpreting the three-dimensional data requires that a three-dimensional model be constructed. As in X-ray crystallography, it is intended that this model represent a simplified view, an abstraction, of the essential information contained in the experimental data. Models from different nuclei in identical biological states can then be compared to search for conserved features [Mathog, 1985]. For the case of nuclei in different states, model analysis can provide quantitative information of the evolution of these states.

Specimen Preparation

All our optical chromosome imaging is done using epifluorescence microscopy and a DNA-specific nonintercalative fluorescent dye (diamidino phenylindole; DAPI). Because this dye binds only to DNA and fluoresces only when bound, it is possible to image chromosomal DNA within intact cells directly; the image is not significantly perturbed by non-DNA-containing cellular components. Furthermore, because the dye does not intercalate, perturbations of the DNA structure and hence chromosomal structure are minimized. *D. melanogaster* early embryos have been chosen because they provide a ready source of synchronized populations of diploid nuclei. The slight mitotic gradient across the embryo provides a spatial mapping of the mitotic cell cycle, simplifying what is in essence a four-dimensional project. For details of buffers and embryo preparation see Hiraoka et al. [1987].

Light Microscopy

Three-dimensional reconstruction of diploid chromosomes via optical microscopy requires that resolutions approaching the diffraction limit be achieved in three dimensions. Ultimately, the quality of an optical three-dimensional reconstruction must reflect the quality of the experimental data used to derive the reconstruction. Fluorescence microscopy, perhaps best among the optical imaging methods, can provide the necessary high contrast and low noise, yet obtaining the data in digital form can be difficult. A combination of very high sensitivity, linearity, and large dynamic range is required to realize fully the information inherent in fluorescent images. Furthermore, because it is necessary to correct for spatial variations in illumination and/or detector sensitivity as well as to deconvolve the recorded images (requiring precise knowledge of image geometry), it is crucial that the recorded images be geometrically and photometrically stable. All these diverse criteria are best met by using cooled, thinned, and back-illuminated CCD imagers [Hiraoka et al., 1987]. These provide quantum efficiencies approaching 0.9 (two to four times the sensitivity of a photomultiplier), very low dark currents (5–50 e, depending on the chip), and very large dynamic ranges (full wells = 300,000–750,000 e). We are currently using an RCA 640 × 1,024 CCD in a Photometrics (Tucson, Arizona) liquid nitrogen-cooled camera. For a detailed characterization of the imaging properties of this CCD, see Hiraoka et al. [1987]. The camera is attached to an inverted Zeiss Axiomat microscope modified so that all aspects of data collection are directly under computer control [Mathog et al., 1985].

Characterization of the Three-Dimensional Optical Transfer Function

To realize fully the intrinsic resolution limits of the light microscope, it is necessary to understand exactly how the recorded image is related to the object under study. For optical sectioning microscopy [Agard, 1984], this requires knowledge of how the two-dimensional contrast transfer function (CTF) changes as a function of defocus. From a family of two-dimensional CTFs, the full three-dimensional CTF of the imaging system can be determined. The two-dimensional CTFs can be calculated from the approximations of Hopkins [1955], Stokseth [1969], or Castleman [1979]. Erhardt et al. [1985] present a direct analytical calculation of the three-dimensional CTF based on the Stokseth approximation.

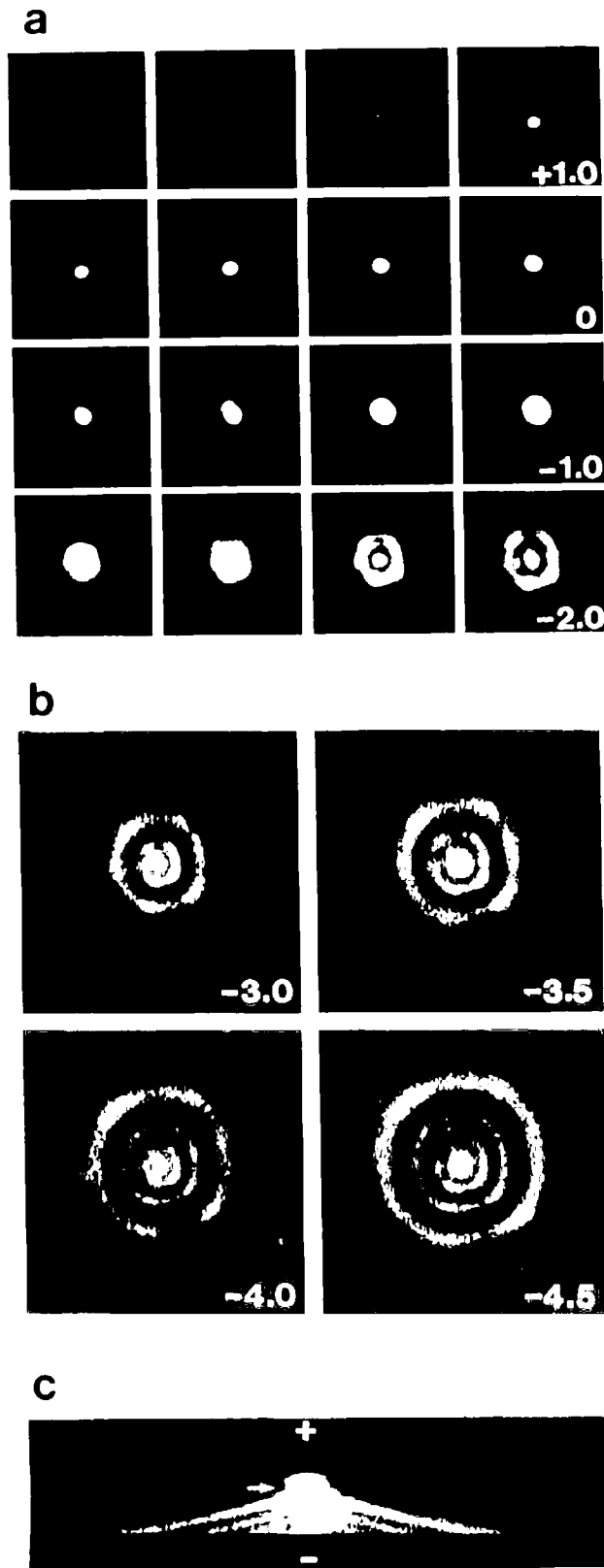
Unfortunately, many assumptions of ideality go into the calculation of the two-dimensional CTFs. The inaccuracy of these assumptions becomes especially pronounced for high-numerical aperture (NA) lenses and becomes greatly exaggerated when calculating three-

dimensional CTFs. Another approach to obtaining the three-dimensional CTF is by direct experimental measurement using very small (< 0.1 μm diameter, smaller than the resolution limit) fluorescent beads to act as point sources. Recently, very high quality CCD imagers have provided the data quality necessary to permit the experimental determination of three-dimensional CTFs of several high-NA objective lenses under epifluorescence conditions [Hiraoka et al., 1987] (also, manuscript in preparation). A set of point images taken at different levels of defocus is shown in Figure 1. It should be apparent that, unlike ideal lenses, the point spread functions (PSFs) of real lenses are not circularly symmetric and can show varying amounts of residual spherical aberration (visualized as lack of symmetry above and below the focal plane). The two-dimensional PSFs in Figure 1 can be radially averaged and Fourier transformed to yield a set of radially symmetric two-dimensional CTFs, which can then be Fourier transformed to yield the three-dimensional CTF (Fig. 2), which can be compared to the theoretical CTF for the same lens. It is important to note that there is a significant difference in behavior of the two CTFs, especially at low-spatial frequencies. Much of the difference results from the fact that the high-NA objective lens serves as a high-NA condenser and provides a partial confocal behavior to the imaging process. Thus we believe that it is extremely important to use the experimental CTF whenever possible. Because there is probably less difference between similar high-NA lenses than there is between experimental and theoretical functions, it is reasonable simply to interpolate from experimental curves to correct for changes expected to result from changing NA and wavelength.

Image Processing

Once a three-dimensional optical section data set has been collected, it is necessary to remove the out-of-focus information that contaminates each image plane. We generally employ one of three processing methods depending on the resolution required. Different types of biological problems can have very different requirements. The simplest method uses two flanking images in a three-dimensional data set to correct the central image. This method works extraordinarily well for a very wide range of problems in cell biology and can essentially be performed in real time using common video processing hardware.

More sophisticated processing requires the calculation of three-dimensional Fourier transforms, which can be very time-consuming unless specialized hardware such as array processors is used. Although the results are definitely better, only the most demanding applications warrant the computational investment.



In many cases, calculation of a completely in-focus projected view of the specimen or an in-focus stereo pair will suffice. Because conventional microscopes provide a partial projection, it is straightforward to turn a stack of images into a true projection or a stereo pair. We call this method "synthetic projection" microscopy because an in-focus projection (or stereo pair) is synthesized from a set of optical sections. Because no light is rejected (either physically as in a confocal microscope or computationally), this method is the least light-intensive way of viewing three-dimensional specimens. As such, it should be ideal for following live cells in near-real time. For a detailed discussion of processing methods see Agard [1984] and Agard et al. [1988].

Experimental Results for Imaging Chromosomes

Much of our laboratories' effort has been devoted to elucidating the detailed high-resolution structure of eukaryotic chromosomes and their spatial organization within the nucleus. Three-dimensional high-voltage and intermediate-voltage electron microscopic tomographic approaches are being used to analyze how the fundamental 100 Å nucleosomal fibers are organized into higher-order structures within mitotic and interphase chromosomes [Belmont et al., 1987]. For some time, we have been using *D. melanogaster* polytene chromosomes as a model interphase system for studying the relationship between three-dimensional spatial organization within the nucleus and biological function [Agard and Sedat, 1983; Mathog et al., 1984; Gruenbaum et al., 1984; Hochstrasser et al., 1986]. The earliest studies were done with images recorded on film and then digitized and aligned before deblurring. The process was greatly simplified with the use of direct video data acquisition. Before and after comparisons of a single section from a data set of 24 focal planes (1.2 μm spacing) of a DAPI-stained salivary gland polytene nucleus are shown in Figure 3. The images were deblurred with the nearest-neighbor scheme.

The results of these studies as well as the development of a growing interest in chromosome dynamic

Fig. 1. Through-focus behavior of point images. Fluorescent microspheres (0.1 μm diameter; Pandex Laboratories, Inc.) were examined using a Leitz 1.4 NA 63× planapo oil-immersion lens with immersion oil recommended by Leitz. The serial focal series was taken with the CCD in 0.25 μm focal steps. In a, the point images from +1.75 to -2.0 μm are shown every 0.25 μm from left to right and top to bottom; -3.0, -3.5, -4.0, and -4.5 μm are shown in b. The x-z edge view of the three-dimensional stack of 31 point images from +3.0 to -4.5 μm at 0.25 μm intervals is shown in c. The in-focus plane is marked by an arrow. All images are displayed on a logarithmic scale to facilitate viewing. [Reproduced from Hiraoka et al. 1987 with permission of the publisher.]

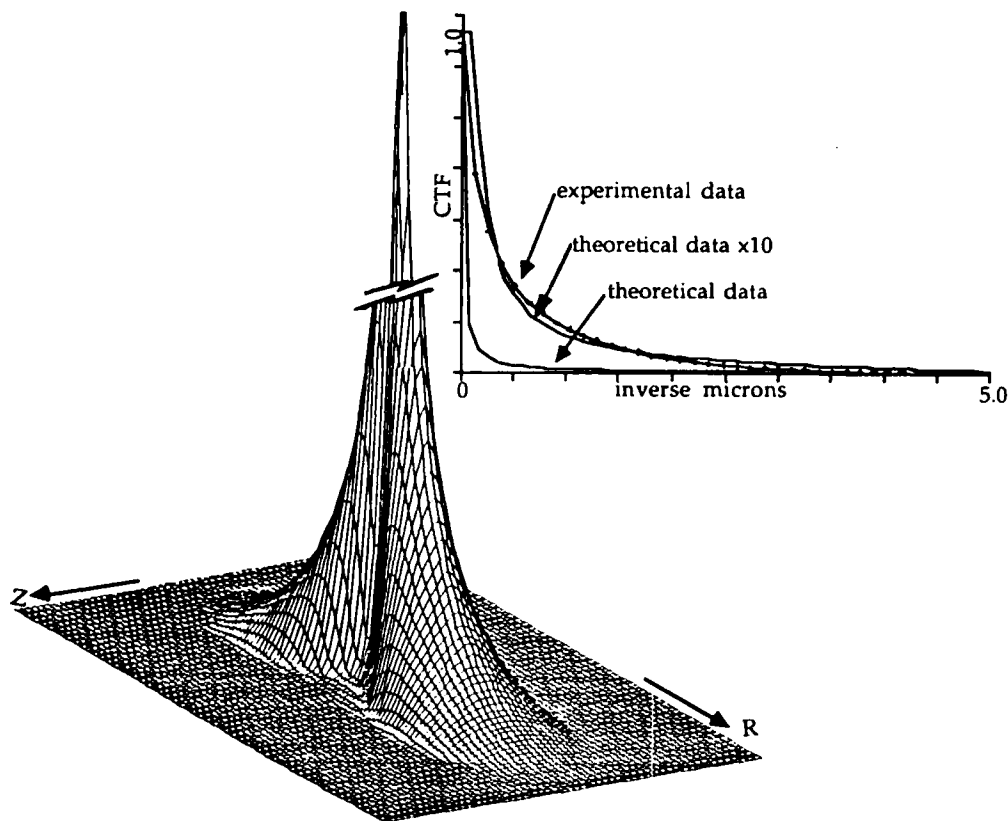


Fig. 2. Experimental three-dimensional CTF from an epifluorescent microscope. Optical section data from individual fluorescent microbeads was collected at $0.2 \mu\text{m}$ intervals using a Zeiss 1.2 NA coverslipless water-immersion lens. Use of this lens greatly minimizes spherical aberration problems, providing a CTF symmetric in Z . Data from several beads within each section image were averaged, three-dimensional Fourier transformed, and cylindrically averaged in Fourier space. The resultant radially symmetrized three-dimensional

CTF is shown (R is the radial term corresponding to the X - Y plane, and Z denotes the focal direction). The inset shows $Z = 0$ lines for the experimental data and from theoretical calculations for the same lens. Note the significantly different fall offs. Even when scaled up tenfold, the theoretical curve still fails to match the experimental data at low resolution. Reproduced from Agard et al. [1988] with permission of the publisher.

behavior during the cell cycle has led us to attempt to analyze the three-dimensional organization of diploid chromosomes as a function of the cell cycle. The comparative difficulty associated with examining a diploid nucleus $5 \mu\text{m}$ in diameter is demonstrated in Figure 3. In comparison with the polytene studies, an increase in three-dimensional resolution of nearly an order of magnitude was required. Several factors were crucial in reaching the necessary resolution; foremost were the use of a cooled CCD to collect very high quality image data and the use of the experimental CTF (CTF determination required the CCD) in the image processing. The substantial improvement in image quality was made possible primarily by using the CCD and secondarily by using a finer interplanar spacing and is shown in Figure 4. Processing again made use of the same simple nearest-neighbor scheme used in Figure 3, which can be implemented on essentially any image-processing system.

High-resolution three-dimensional images of dip-

loid chromosomes from *D. melanogaster* early embryos are shown as several optical sections before and after image processing in Figure 5. A stereo pair computed from the entire nucleus is shown in Figure 6. Stereo pairs calculated from another diploid *Drosophila* nucleus are shown in Figure 7. A three-dimensional model built to the same three-dimensional stack of data is also shown as a stereo pair in Figure 7. The sister and homologous chromatids are clearly separated; the bright regions represent clusters of centric heterochromatin. The three-dimensional paths of each of the chromatids have been traced using our interactive modeling software [Mathog, 1985]. This is the first step in our analysis of the dynamic behavior of diploid chromosomes throughout the cell cycle.

Once a model describing the three-dimensional path of a chromosome has been built, it is then possible to use that model as a map for topologically unfolding the chromosome. This is extremely useful to us, because

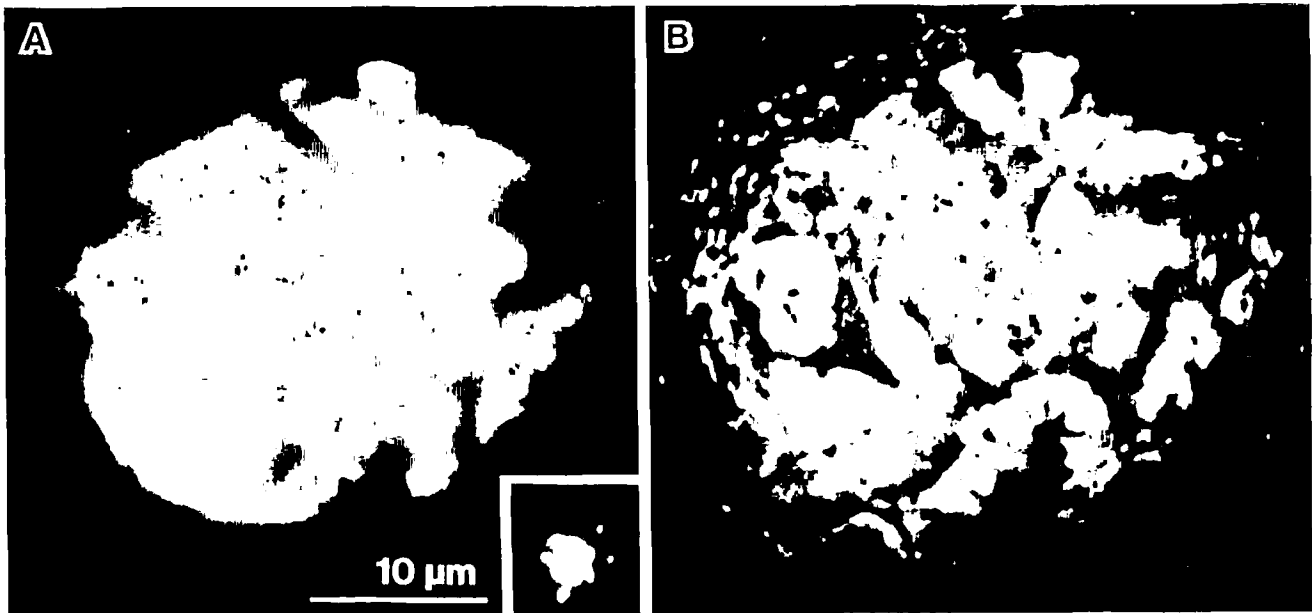


Fig. 3. Low-resolution polytene chromosome images before and after processing. *Drosophila* salivary glands were stained with the DNA-specific dye DAPI as described in Mathog et al. [1984]; 24 images were recorded at 1.2 μm focal intervals using an SIT camera, and each image was digitally averaged for 256 video frames. The nearest-neighbor algorithm [Gruenbaum et al., 1984] was used to

process the images. A representative before section is shown in A and the corresponding after processing image is shown in B. Puffed regions indicating intense transcriptional activity are clearly seen in B. The inset in A shows a *Drosophila* embryonic diploid nucleus for comparison. Reproduced from Agard et al. [1988] with permission of the publisher.



Fig. 4. High-resolution polytene chromosome images before and after processing. Polytene chromosomes were stained with DAPI and imaged on our cooled CCD system [Hiraoka et al., 1987] with an

interplanar spacing of 0.5 μm . Data were processed as in Figure 3. Note the significantly higher resolution possible using the CCD to collect better data and finer interplanar spacings.



Fig. 5. Embryonic diploid nucleus before and after the removal of out-of-focus image information. *D. melanogaster* embryos stained with 0.1 μM DAPI were attached to a glass slide and mounted in buffer A without a coverslip. Using a Zeiss coverslip-free water lens ($63\times/\text{NA } 1.2$), optical section images were taken on the CCD at 0.25 μm focal intervals. Optical section images were processed by the iterative constrained three-dimensional deconvolution method [Agard et al., 1988] with the experimental CTF determined for the water lens. This figure shows the same nucleus shown in the inset of Figure 3 before and after processing; each section in the figure is separated by 1 μm in z direction. Reproduced from Agard et al. [1988] with permission of the publisher.

it allows us to identify uniquely each chromosome arm using its characteristic staining patterns. An example of this is shown in Figure 8.

SUMMARY

The combination of the specificity provided by fluorescence microscopy and the ability to analyze specimens quantitatively in three dimensions allows the fundamental organization of cells to be probed as never before. Key features in this emergent technology have been the development of a wide variety of fluorescent

dyes or fluorescently labeled probes to provide the requisite specificity. High-quality, cooled CCDs have recently become available. Functioning as nearly ideal imagers or "electronic film," they are more sensitive than photomultipliers and provide extraordinarily accurate direct digital read-out from the microscope.

Not only is this precision crucial for accurate quantitative imaging such as that required for the ratioing necessary to determine intracellular ion concentrations, but it also opens the way for sophisticated image processing. It is important to realize that image processing is not simply a means to improve image aesthetics but can directly provide new, biologically important information. The impact of modern video microscopy techniques [Allen, 1985; Inoué, 1986] attests to the fact that many biologically relevant phenomena take place at the limits of conventional microscopy. Image processing can be used to enhance substantially the resolution and contrast obtainable in two dimensions, allowing the invisible to be seen and quantitated.

Cells are intrinsically three-dimensional. This can be simply a nuisance because of limited depth of focus of the microscope, or it can be a fundamental aspect of the problem being studied. In either case, image processing techniques can be used to provide rapidly the desired representation of the data. In this paper, we discuss the nature of image formation in three dimensions and deal with several means to remove contaminating out-of-focus information. The most straightforward of these methods uses only information from adjacent focal planes to correct the central one. This approach can be readily applied to virtually any problem using standard image processing hardware to provide a substantially deblurred image in almost-real time. In addition to designing more sophisticated algorithms in which the utmost in three-dimensional imaging is required, we have developed a method for extremely rapidly and accurately producing an in-focus, high-resolution "synthetic projection" image from a thick specimen. This is equivalent to that produced by a microscope having the impossible combination of a high-NA objective lens and an infinite depth of focus. A variation on this method allows efficient calculation of stereo pairs.

Because these synthetic projection methods are both computationally efficient and efficient in their use of the fluorescent light emitted from the sample, they are ideally suited for examining living cells or when using fluorophores that are very sensitive to photobleaching. It should now be possible to generate complete stereo pairs at a rate of one every 15–20 sec. Hardware improvements expected within the year as well as making some slight concessions to image quality should reduce the time per stereo pair to about 2–3 sec. This should be sufficiently fast to allow complex and dynamic biologi-

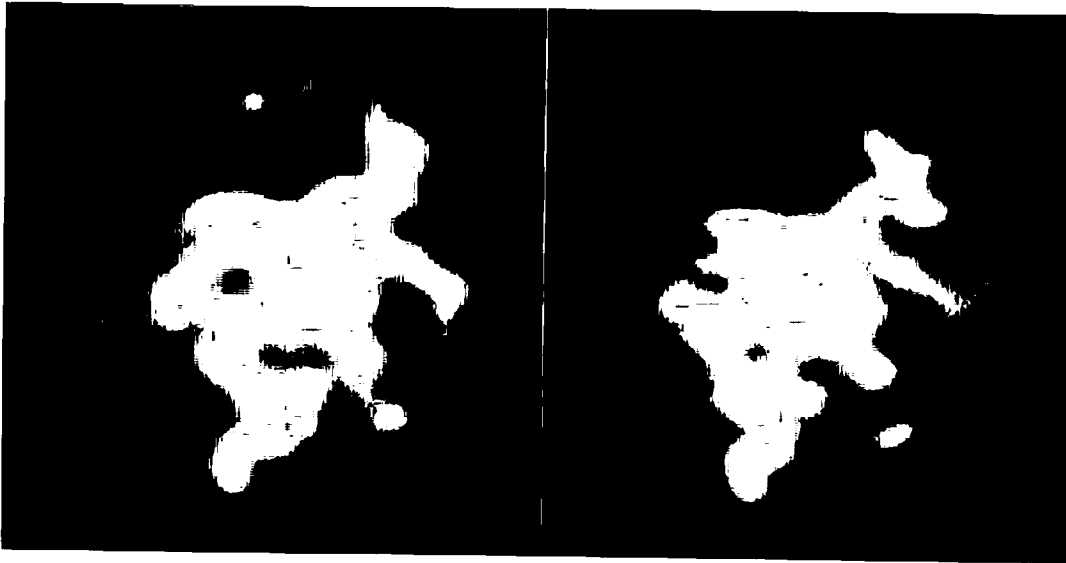


Fig. 6. Stereo image of diploid embryonic chromosomes. A stereo projection was constructed from the optical sections shown in Figure

5 after deblurring using the rotated-projection method. Reproduced from Agard et al. [1988] with permission of the publisher.

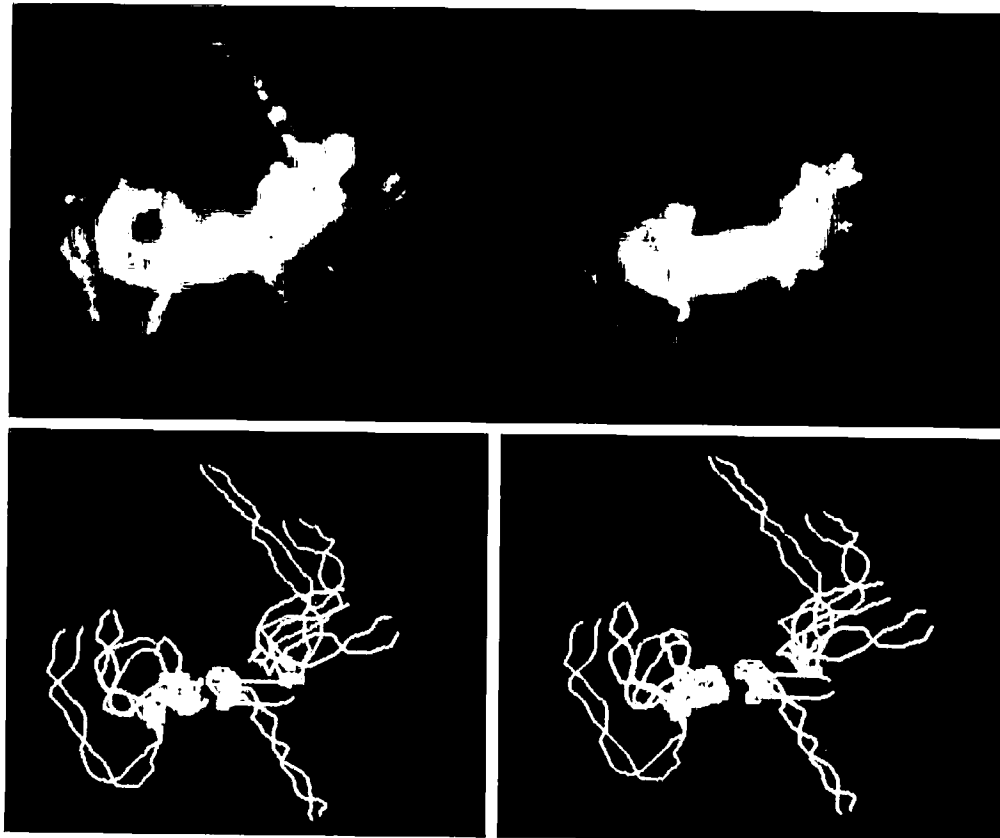


Fig. 7. Stereo model of the three-dimensional spatial arrangement of diploid chromosomes. The stereo image was constructed from optical section images taken with $0.1 \mu\text{m}$ step size in z . Out-of-focus information was removed as in Figure 5. Chromosome paths were

traced in the stack of optical sections using our interactive modeling software [Mathog, 1985]. Each line represents a sister chromatid; the central bright area is the centric heterochromatin cluster. Reproduced from Agard et al. [1988] with permission of the publisher.

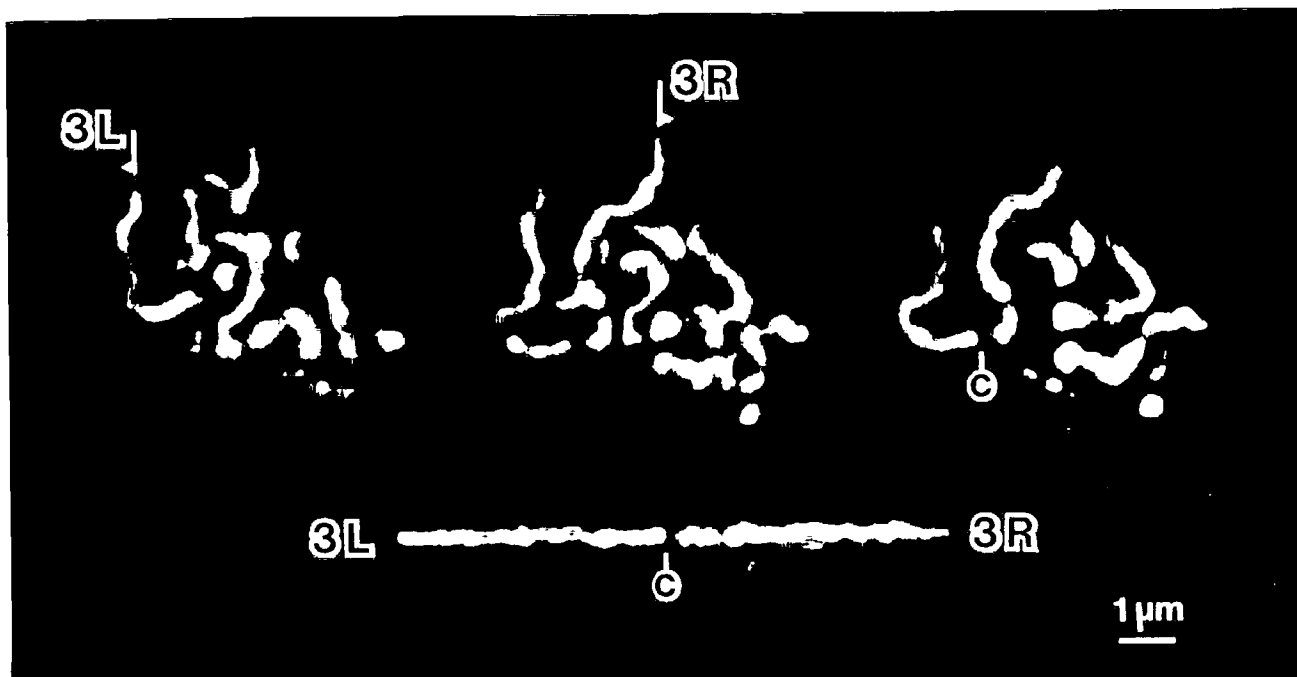


Fig. 8. Cytological assignment of diploid chromosomes. Gently squashed embryos were imaged at $0.5 \mu\text{m}$ focal intervals, using a Leitz oil lens ($63\times/\text{NA } 1.4$). Immersion oil with the refractive index of 1.518 (Cargille Laboratory, Inc.) was used to minimize spherical aberration (results comparable to using the Zeiss 1.2 NA coverslipless

water-immersion lens). Chromosome paths were traced, and then the traced chromosomes were computationally straightened using the modeled chromosome path. This figure demonstrates the straightening of the third chromosome. C, centromere. Reproduced from Agard et al. [1988] with permission of the publisher.

cal phenomena to be studied in three dimensions in living cells.

Although our studies have focused on the three-dimensional organization of chromosomes within the nucleus, it should be apparent that the methods being used are quite general and are suitable for many problems in structural cell biology. In our own laboratories, we have begun collaborations with colleagues at UCSF to study polarization phenomena in epithelial cells and spatial aspects of regulated transport through the golgi. With the development of processing, display, and modeling software, it is now possible to determine quantitatively, to analyze, and to compare three-dimensional distributions of cellular components.

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