SPATIAL ORGANIZATION OF THE DROSOPHILA NUCLEUS: A THREE-DIMENSIONAL CYTOGENETIC STUDY

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SUMMARY

The combination of optical fluorescence microscopy with digital image processing and analysis has been used to examine the three-dimensional organization of chromosomes within intact polytene nuclei. Although the arrangement indicates a high degree of flexibility, there are many conserved features between nuclei at the same developmental stage. For example, chromosome arms are loosely coiled with centromeres clustered at the opposite end of the nucleus from the telomeres. Individual chromosome arms are not interwoven but occupy different spatial domains. Chromosomal sites that contact the envelope correlate with intercalary heterochromatin. Connections are observed between actively transcribing regions.

INTRODUCTION

Optical section microscopy uniquely allows the analysis of the three-dimensional arrangement of cellular and sub-cellular components in intact tissues. As part of a continuing investigation into the structure and function of eukaryotic chromosomes, we have used this powerful method to map the spatial organization of the giant polytene chromosomes in Drosophila melanogaster salivary gland nuclei. Although aspects of this work have already been reported (Agard & Sedat, 1983; Mathog et al. 1984), in this paper we present the conclusions obtained from an analysis of chromosomal folding in 10 nuclei as well as preliminary findings on both the spatial association of transcriptionally active regions and the correlation between transcriptional activity and three-dimensional location. In addition we report on the use of improved algorithms for removal of out-of-focus information during the analysis of optical section images.

DATA COLLECTION AND ANALYSIS

Although details of the preparation of glands for microscopy (Mortin & Sedat, 1982; Sedat & Manueldis, 1977) and the computer-controlled microscope and data collection hardware (Mathog, Hochstrasser & Sedat, 1985) are described elsewhere,

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the salient features will be briefly mentioned. Salivary glands from late third-instar *D. melanogaster* larvae are carefully hand-dissected into a buffer optimized for the preservation of chromosome structure (Sedat & Manuelidis, 1977) and subsequently stained with the DNA-specific, non-intercalative fluorescent dye DAPI (4', 6-diamidino-2-phenylindole). By using such a fluorescent dye, only the DNA-containing structures are imaged, largely unencumbered by proteinaceous or membrane material. Using this approach it is possible to examine chromosome organization in intact cells located within a whole salivary gland.

Fluorescent images were recorded using a 100× 1.3 N.A. oil-immersion lens on an inverted Zeiss Axiomat microscope set up for epi-fluorescence. Under computer control, the focal plane was increased by small amounts (1.2–1.4 µm), and pixel 512×512 images were acquired from a SIT video camera and digitized. At each focal position, 256 digital images were averaged (each taken in 1/30 s) to reduce noise. A typical data set consisted of 24, 512×512 digital images.

Because of the finite depth of focus of the microscope's objective lens, each image represents the sum of in-focus information from a narrow plane within the specimen and out-of-focus information from the remainder of the sample. From a knowledge of the specific objective lens parameters, it is possible to determine the precise relationships between the true three-dimensional image and what is actually recorded by the microscope. A comprehensive discussion of the image-forming properties of a microscope as well as an analysis of reconstruction algorithms is presented elsewhere (Agard, 1984; Castleman, 1979). Because of the rather coarse sampling used here, relatively simple approaches can substantially remove the out-of-focus component that contaminates each image.

The approach chosen is a modification of that of Weinstein & Castleman (1971). For any section *j*, the reconstructed image *ij* is calculated from the observed section *oj* and the ones immediately above it *oj+1* and below it *oj−1*. The adjacent sections are first blurred by an amount corresponding to one focal step, scaled by an empirically determined constant and subtracted from the current section. The resultant image is then de-blurred by the in-focus contrast-transfer function in order to sharpen the image. For simplicity and ease of calculation these operations are all done in Fourier space:

\[ I(u,v) = (O(u,v) - s_1(r)[O(u,v)_{j−1} + O(u,v)_{j+1}])/s_0(r), \]

where \( I(u,v) \), \( O(u,v) \) are the two-dimensional Fourier transforms of the observed \( o(x,y) \) and final \( i(x,y) \) images, \( r = (u^2 + v^2)^{1/2} \). The in-focus contrast-transfer function \( s_0(r) \) and the out-of-focus contrast-transfer function \( s_1(r) \) are calculated from the theory of Stokseth (1969) as described by Castleman (1979) and shown in Fig. 1. The final enhanced image stack is calculated by inverse Fourier transformation of the \( I(u,v) \) values. Examples of several sections before and after processing are shown in Fig. 2. The improvement in image quality due to de-blurring greatly facilitates the reading of cytology. This is especially true of glands from other developmental stages that are less transparent and show more light scattering. However, the data discussed in this communication were collected from nuclei which were sufficiently clear that no image processing was required.
Fig. 1. A family of calculated contrast transfer functions (CTF) is shown for a 100× 1.3 N.A. Planapochromat oil-immersion objective lens. The degree of defocus (in μm) is shown by each curve.

**EVALUATION OF CHROMOSOME ORGANIZATION WITHIN THE NUCLEUS**

The initial aim of this work was a description of the manner in which polytene chromosomes are packaged within the cell nucleus. Because the three-dimensional folding pattern of the chromosomes within the nucleus is neither simple nor identical between different nuclei, it has been necessary to find objective methods for evaluating these structures and for assessing the degree of similarity between different nuclei. What is desired is the ability to quantitate and compare the functionally significant aspects of structural organization. Unfortunately, it is by no means obvious what the most relevant structural description might be. Our approach has been to compare a set of nuclei in a single tissue at a specific time in development and determine if any regularities can be recognized, the assumption being that similarities reflect functionally important structural aspects of the nucleus (Mathog *et al.* 1984). This work represents an extension of the original data set (Mathog *et al.* 1984) to ten nuclei.

The three-dimensional chromosomal layout can be studied at several levels of detail. First of all, the optical section data set itself can be inspected. It is possible to draw several broad conclusions based on such observations. First, the bulk of the chromosomes are found in the peripheral shells of the nuclear volume, leaving a
Fig. 2. Two optical sections from a stack of 24 are shown before (A,C) and after (B,D) processing to remove out-of-focus information. Note that the fine banding detail is considerably more apparent in the processed images. After processing, some of the intrinsic aberrations in the current SIT video camera also become more apparent. The use of better detectors will avoid this problem. These data come from a gland which has been fixed and embedded in Spurr and subsequently optically sectioned.

central zone largely free of chromatin. Second, the chromosome pathway does not always trace a smooth curve but is marked by a number of kinks. These sharp changes
in direction are found most frequently at chromosomal weak points or at puffs. Finally, certain regions are marked by an unusual degree of contortion, and in favourable circumstances can be seen to give rise to ectopic fibres (for a discussion on ectopic fibres see Kaufmann & Iddles, 1963).

The complicated folding of chromosomes and the wealth of detail available in the images demands the abstracting of this information into a more readily analysable form. A software package has been created (Mathog et al. 1985) that allows the pathways of the different chromosome arms to be traced out within the three-dimensional data set. By careful examination of the optical section data it has been possible to recognize the characteristic banding patterns revealed by fluorescence staining. With reference to maps made from squashed preparations, precise cytogenetic locations can be assigned along the chromosome path. The resulting stick- figure model, complete with cytogenetic information (an example is shown in Fig. 3), is then examined for various folding features. The analysis at this level has revealed additional insights into nuclear organization. (1) The most common folding motif is the loop. Chromosome arms often coil into three to four successive gyres, and these irregular helices are almost always right-handed. (2) The centromeres are aggregated into a single chromocentre, which is invariably in contact with the nuclear envelope. (3) The chromosome arms do not usually fold into tight globular units but are more or less extended, with telomeres at the opposite end of the nucleus from the chromocentre. This is the classical Rabl orientation (Rabl, 1885) noted previously in diploid cells, particularly those of plants (Fussell, 1975). (4) Most surprisingly, the chromosome arms are each restricted to a different region of the nucleus; no interwoven arms have ever been observed in salivary gland nuclei.

Structural information can be further abstracted from the three-dimensional chromosome models in the form of quantitative plots. We have tested a variety of rotationally invariant structural parameters and have used them to quantitate various organizational features (Mathog, 1985). One means of representing three-dimensional data in two-dimensional form is the intradistance diagonal plot, which has been used extensively in evaluating protein crystal structures (Rossman & Liljas, 1974). Fig. 4 shows a representative diagonal plot derived from chromosome arm 3L in one of the reconstructed nuclei. Each axis represents cytogenetic position along the chromosome, beginning in each case with the centromere at the origin. The graph depicts a contoured set of distances displayed as intensity values, the brighter values representing chromosome points that are close together in space. The contour steps are 1 μm; any intradistance value greater than 8 μm is set to black. The distances between any two cytogenetic points on the chromosome can be found by locating each point on the x and y axes, respectively, and finding the intersection of the orthogonals.

Fig. 3. Stereo pair of a model derived from one polytene nucleus. There are five major chromosome arms: X (green), 2L (orange), 2R (blue), 3L (purple) and 3R (light green). The centromere of each is marked by a square while each telomere is denoted by a triangle. The centromeres are actually aggregated to form a single chromocentre; the telomeres are located in a more dispersed manner at the opposite end of the nucleus (Rabl orientation).
Fig. 4. An intradistance plot of the 3L chromosome from one nucleus. Each axis represents a cytological position, starting with the centromere at the lower left; 20–30 bands from the cytogenetic sequence have been used to normalize the plot onto a standard grid. Each subdivision of the grid represents two divisions (out of 20) on the cytological map (Bridges, 1935), with the telomere, t, 61A at the two extrema and of the chromocentre, cc, 80F at the origin. Intensities, which are inversely proportional to the absolute distance between any two chromosomal points, are contoured in steps of 1 μm. Chromosome points further apart than 8 μm are shown in black.

drawn from them. The intensity value at this position is inversely proportional to the absolute distance between the two loci.

These intradistance maps are used to assess the folding of each arm and to facilitate the comparison of corresponding arms in different nuclei. The loop motifs mentioned above can be seen in the plot in Fig. 4 as bars of intensity extending at right angles from the diagonal. Their position within the cytogenetic sequence is now easily determined. This work is still in a preliminary stage and further details will be published elsewhere.

In attempting to compare chromosome structures in different nuclei two principal problems have been encountered. Most importantly, we have found it necessary to have a set of guide-marks along each chromosome to ensure that precisely corresponding regions are compared. As it is possible to follow the banding pattern in situ, this requirement is readily met. The second general difficulty is in the quantitative
comparison itself. Only a moderate number of nuclei have been reconstructed so far, and the chromosome packaging is sufficiently flexible to make it difficult, in some cases, to distinguish statistical noise from significant differences. A rank order parameter has previously been used to compare the diagonal plots from multiple nuclei (Mathog et al. 1984). With the increase in the number of data sets, other statistical methods are now possible.

A particularly informative quantitative measurement has been the minimum distance between the nuclear envelope and loci along a chromosome (Mathog et al. 1984). This revealed a set of highly conserved ‘contacts’ with the nuclear envelope. We have expanded the initially reported data set by another four nuclei from the same salivary gland, and essentially all of the loci identified previously as conserved nuclear envelope contact sites remain so classified. Fig. 5a shows the superimposed plots of 10 2L chromosomes; in b the average distance to the surface is plotted, along with the standard deviations. As seen previously, the correlation of these surface contact sites with regions of intercalary heterochromatin (loci with high frequencies of ectopic pairing and rearrangement, and which have late-replicating properties shared with centric heterochromatin; Zhimulev, Semeshin, Kulichkov & Belyaeva, 1982) is very strong.

**PRELIMINARY ANALYSIS OF TRANSCRIPTIONALLY ACTIVE LOCI**

In an effort to better understand the significant functional properties of chromosomal organization, we have begun a series of experiments to probe the correlation of

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Fig. 5. The distance-to-surface plots of ten 3L chromosomes. A. The 10 plots superimposed. The abscissa is the same as for Fig. 4. The ordinate represents the minimum distance to the nuclear surface and is scaled to 5 μm per division, starting at 0 for each plot. Points on the curves that touch the x axis are loci that are at the surface of the nucleus. Peaks on the curves denote chromosomal points that extend furthest into the nuclear interior. B. The average of the 10 plots, shown within an envelope of ±1 standard deviation.
structure with transcriptional activity. We have directly analysed the three-dimensional
distribution of actively transcribing DNA in salivary gland nuclei by labelling the DNA
with the fluorescent dye DAPI and the nascent RNA with a short pulse of tritiated uridine.

Salivary glands were again hand-dissected from third instar larvae and pulsed for
4 min in modified Shield & Sang (1970) medium that contained the RNA precursor
$[5^3$H]uridine (29 Ci/mmol). Following fixation with formaldehyde and per-
meabilization with Triton X-100, the glands were stained with DAPI, refixed in
formaldehyde, embedded in Spurr's resin and serially sectioned. The 0.7 μm thick
sections were prepared for autoradiography and exposed for 12 days. Images of both
the fluorescent chromosomes and the silver grains from each section were digitized and
aligned, using the algorithm of Agard & Sedat (1980), to form two registered
image stacks. Using this approach, and our interactive modelling programs (Mathog,
et al. 1985), we have been able to reconstruct two complete nuclei from physical
sections. In both cases, the paths of the chromosomes complete with their cytogenetic
markers have been determined.

Since only two nuclei have been examined, any conclusions drawn at this point are
necessarily very speculative. However, two very interesting observations have been
made. First, it appears that the spatial distribution of active loci is very heterogeneous.
That is, there are regions of the nucleus in which highly active transcribed regions are
located, as well as regions relatively devoid of transcriptional activity. In addition,
there seems to be some correlation between intense transcriptional activity and
proximity to the nuclear membrane, as there is between chromosome position and
proximity to the envelope. This remains to be analysed rigorously.

Another interesting observation from the pulse-labelling experiments is that con-
nections of silver grains between spatially close puffs are frequently seen. These
extensions from the edge of the puff are decidedly asymmetric, and are only seen on
the edge that is adjacent to another puff. This suggests that there might be some link
between active regions on different chromosomes or between different regions on the
same chromosome (Fig. 6a,b).

The existence of RNA connections suggested by the pulse-labelling experiments is
strongly supported by experiments using another approach to label active genes.
Whole salivary glands have been stained by indirect immunofluorescence using
monoclonal antibodies X4 or S5 that had previously been shown to be specific for
different classes of ribonucleoprotein (RNP) particles (Risau, Symmons, Saumweber
& Frasch, 1983; Saumweber et al. 1980). Following staining with rhodamine-
conjugated second antibody, the chromosomes were stained with DAPI. Nuclei
prepared in this manner were either physically or optically sectioned. Results from
physical sectioning of an embedded gland labelled with the X4 antibody are presented
in Fig. 6c,d. Similar results were obtained using the S5 antibody. Connections
between different chromosome loci that have these RNP antigens can be seen at many
locations within each nucleus using either antibody. Similar fluorescent antibody
connections can be viewed in optical sections of whole glands as well as in isolated
nuclei from the same tissue (data not shown). The equivalence of the antibody and
grain connections is now being rigorously established.
Fig. 6. A, B Salivary glands were pulse-labelled with [5-3H]uridine for 4 min as described in the text. The DAPI-stained image of a section is shown in A and the autoradiography image in B. C, D. Fluorescent images of the DNA and hnRNP antigens, respectively, from the same physical section. The antibody used here was X4. Some of the connections here are indicated by arrowheads.

Great care has been taken to ensure that these connections links are not preparation artifacts. Several different regimes of fixation and sample preparation have been tried, and in all cases connections were seen. Labelling with other monoclonal antibodies that are not directed against heterogeneous nuclear RNP (hnRNP) antigens, which also stain multiple sites along the polytene chromosome, did not give rise to such connections.

**Conclusions and Speculations**

Several features of the present results are particularly striking. First is the fact that
the polytene chromosome arms remain, in most cases, in the polarized Rabl orientation. This configuration is seen in dividing cells and is thought to result from the traction of the previous anaphase movement (Fussell, 1975). The salivary gland nuclei, however, underwent their last division early in embryogenesis, yet they continue to maintain their polarized chromosome configuration. A precedent for this long-term positional stability is the finding by Sperling & Luedke (1981) that quiescent muntjack lymphocytes also retain the Rabl orientation for extended periods of time. However, in the case of the polytene chromosomes the result is still surprising as there have been some 10 rounds of DNA replication, during which time the mechanical properties of the chromosomes as well as the physical properties of the nuclei might be expected to change considerably.

The presence of topological boundaries observed between chromosomes is an even more unexpected example of long-range positional stability. It is somewhat reminiscent of the separation of individual chromosomes into vesicles or karyomeres seen in the early stages of nucleus reformation in rapidly dividing embryonic cells (Ito, Dan & Goodenough, 1981; Wilson, 1925). It is possible that nuclear architectural elements such as the matrix may remain organized in subnuclear domains that isolate each chromosome. In any case, the simplest interpretation of the absence of intertwining of different arms seems to be that they too reflect the positions of the preceding mitotic chromosomes. For dividing nuclei, this would allow the straightforward condensation of each chromosome arm during the transition from interphase to metaphase.

The chromosomal sites that appose the nuclear envelope in all or virtually all the nuclei have been shown to correlate very well with regions of intercalary heterochromatin (IH). As argued previously, these sites may also serve as envelope–chromosome anchorage sites (Mathog et al. 1984). It is worth pointing out, however, that there are many more IH loci than conserved envelope contact regions; in other words, there are many 'unused' anchorage loci. Since it is known that centric heterochromatin can be arranged differently in nuclei of different cell types (Hsu, Cooper, Mace & Brinkley, 1971), it is conceivable that the other IH loci are used as anchorage points in other tissues, generating alternative nuclear arrangements of chromosomes. Perhaps in different tissues different functional requirements may be met by a different chromosomal architecture. Several other tissues in D. melanogaster are sufficiently polynized to test this hypothesis, and such work is under way. It should be noted that when Zhimulev et al. (1982) compared IH characteristics in different tissues, the IH loci all mapped to the same sites as found in salivary glands.

The striking observation of connections between actively transcribing regions suggests a possible metabolic link between heterologous chromosome sites. Whether this represents a sharing of transcriptional or RNA-processing machinery, or has a structural or regulatory role, or is merely fortuitous is, as yet, unclear. Any functional purpose would have to be linked to the three-dimensional folding of the chromosomes inside the interphase nucleus. Further experiments to clarify this point are now under way.

All of the studies reported here involved relatively few sections separated by rather
large intervals. We are currently exploring the use of considerably higher-resolution data sets (128 images, each separated by 0.3 µm). The hope is to be able to extend these studies to diploid prophase and possibly interphase nuclei. It should also be possible to examine directly ectopic fibre locations in intact salivary gland nuclei. In addition to using more data, it is essential that more-accurate reconstruction (out-of-focus removal) algorithms are used. Two methods that appear to provide the requisite accuracy have been analysed in detail (Agard, 1984) on mock data and are now being tried on real data.

In addition to providing new insights into chromosome architecture, these powerful new approaches for studying structure at the cellular level in intact tissues should prove quite useful in other areas of cell biology. Possible areas include: analysis of spatial organization of actin or tubulin, or secretory components, or possibly even neuron interconnections within the nervous system.

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