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The Homing Cursor: A Tool for Three-Dimensional Chromosome Analysis

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When studying the three-dimensional shape of prophase chromosomes (or any other tubular structure), it is useful to represent these structures as a string of three-dimensional Cartesian coordinates along the medial axis. This procedure was automated in order to limit the number of human interactions and to improve reproducibility. In this paper the design, implementation, and validation of the automated method is presented. From the data presented it can be concluded that the cursor algorithm provides an objective and therefore reproducible method to trace the medial axes of prophase chromosomes automatically. This method could allow a more extensive understanding of the (changes in) chromosome organisation throughout the cell cycle, its relation to cell function, and the complex process of chromosome condensation.

Key terms: 3-D image processing, tubular shape, automated tracing

Analysis of the three-dimensional (3-D) organisation of the eukaryote cell nucleus has become possible as a result of the availability of confocal microscopes and the parallel development of 3-D image processing techniques (3,4,19,20). A basic rationale in this field of research is the assumption that the expression of genes not only depends on molecular regulatory units, but that it is also related to the 3-D spatial organisation of genes and chromosomes within the cell nucleus (2).

Recent advances in fluorescent in situ hybridization (FISH) techniques have enabled the visualisation of individual genes (10,18), selected chromosomal domains (5,12), and entire single chromosomes in interphase nuclei (11,17). Many of the findings suggest particular arrangements in 3-D chromatin organisation (for a review, see ref. 13), although it remains difficult to establish chromosome organisation fully in interphase.

A different approach to this matter, which may contribute to unravelling the secrets of the interphase nucleus, is to investigate the 3-D folding pattern of individual prophase chromosomes (9,15). It has been argued that in early stages of prophase, that is, when chromatin condensation just has started, the 3-D position of chromosomes can be extrapolated to chromosome organisation in interphase. The analysis of chromosome shape may then provide important information with respect to (cell type related) regularities in positioning of chromosomes and genes in interphase as well as to the mechanisms underlying chromatin condensation.

When studying the spatial arrangement of a tubular prophase chromosome it is useful to represent it by its medial axis. These representations have been used by Agard and Sedat (1,7,8,14) in their study of Drosophila polytene interphase chromosomes. 3-D coordinates of points along the chromosome axes were obtained by interactively indicating them with a cursor in 2-D slices of the 3-D image. Apart from the time it takes to perform such a procedure, the potential disadvantage of a method based on human interaction is that it involves many difficult, often ambiguous, decisions. In previous communications we reported a semi-automated method, in which an objective measure for finding the medial axis was introduced (9). Although this method is an improvement, the number of human interactions is still high.

This paper describes the design, implementation,
and validation of an automated method for the description of the 3-D shape of prophase chromosomes.

MATERIALS AND METHODS

Description of the Cursor Program

The homing cursor is a computer program used to obtain strings of 3-D Cartesian coordinates of points along the medial axis of prophase chromosomes or other tubular structures in a 3-D data set. The representation of an (unbranched) tubular structure as a string of coordinates serves three goals: 1) it reduces the vast amount of data containing a 3-D image to a string of coordinates; 2) it enables real time rendering by graphics representation; and 3) it facilitates such mathematical calculations as curvature and torsion measurements.

The procedure for obtaining these 3-D coordinate strings has two parts. The first is interactive: the user provides essential input for the second, automatic part, in which the medial axis of a chromosome is traced automatically from one end to the other.

In the interactive part a 3-D cursor is used to indicate a starting point, a starting direction, and an end point. The cursor is present in three windows on the computer screen (Fig. 1a). The central window displays a front view (XY projection) of the 3-D confocal data set. The windows on the side display a top view (XZ projection) and a side view (YZ projection). In this way, the user accurately indicates the required start and stop positions within the 3-D image. In Figure 1a and b, the cursor is interactively positioned at the starting and stopping point; in Figure 1c it has automatically traced the selected chromosome.

In the automatic part, the cursor starts at the user-defined starting point and goes along the medial axis of a chromosome in equidistant steps (Figs. 1c, 2a). It stops as it reaches the near vicinity of the user-defined stop point. When the chromosome is traced with the use of kernel type 1, the cursor gets trapped in these areas (the reason for this is clarified in Fig. 3c). To avoid this problem, in this case kernel type 2 was used with sizes $m_r = 3$ (width) and $m_z = 6$ (length).
end point. The algorithm is based on the assumption that in a 3-D confocal image, a prophase chromosome can be considered to be a tube with higher light intensities near its medial axis and lower ones away from the axis. From its current position, the algorithm searches in its current forward direction for a local maximum of high light density in its near neighbour- hood. The 3-D coordinates of the points within the image encountered in this way are stored in the computer memory.

**Description of the Tracing Algorithm**

The tracing algorithm functions on the basis of four principles: 1) at each step, a limited set of directions is considered; 2) to select the best direction, the light density at a point in that direction is evaluated; 3) for sufficient accuracy, the cursor is allowed to step "in between voxels"; and 4) the direction to go to finally is obtained by interpolation of the selected best direction and its direct neighbours within the limited set.

The symbols used in the following description are summarized in Table 1.

1. At each step a limited set of directions is taken into consideration. At the current position, $P_c$, the algorithm starts by evaluating the current (forward) direction, $d_e$. At the first step this is the user-defined starting direction $d_s$. Next, the algorithm considers a limited number of directions $d^e$, with the same small angle $\phi = h_s$ with the current direction and an angle $h_a$ with one another. These directions form a "ring" (Fig. 2b).

2. For each direction, the light density of the image at a point in that direction at distance $h$ (the lateral grid size) from the current position is calculated. At this point the image is convoluted with a Gaussian kernel. Two kernel types have been considered. The first kernel is a spherical Gaussian function (Fig. 3a,b). The weight factors are calculated as: $k(x,y,z) = A \cdot \exp(-c^2 d^2/m_r^2)$, where $c^2 = x^2 + y^2 + 2z^2$, the distance to the centre of the kernel $m_r$ defines the radius of the kernel, and $A = 255/m_r \cdot 2\pi$. The second kernel is a tubular Gaussian function (Fig. 3c,d). The values of the weight factors depend on the closest distance to the axis of the tube in a Gaussian way. The weight factors are calculated as: $k(x,y,z) = A \cdot \exp(-d^2/m_t^2)$, where $d^2 = \sqrt{x^2 + y^2}$, the closest distance to the axis of the tube.

Given $P_c$ and $d_e$, the convolution is calculated for each $\phi$ and $\theta$ as:

$$g(\phi, \theta) = \sum_{x=-m}^{m} \sum_{y=-m}^{m} M[M \cdot (x,y,z)] \cdot k(x,y,z),$$

where $M$ is a matrix translating $[x,y,z]$ over $P_c$, and rotating the result about $d_e$ over $\phi$ and $\theta$, $f_i$ is the data array of trilinearly interpolated image grey values, and $m_r$ and $m_t$ determine the radius and length of the kernel.

If $g_{\text{max,1}}$ is the highest convolution result found in ring 1, is less than the maximum in the current direction, the current direction is selected to be best direction. If not, ring 2, with $\phi = 2h_a$, is evaluated (note that the successive rings together constitute the surface of a sphere (Fig. 2b)). If $g_{\text{max,2}} < g_{\text{max,1}}$, the direction, $d_{e,e}$, that is, the direction corresponding to $g_{\text{max,1}}$ is selected, and so on.
Summary of the Symbols Used in the Description of the Algorithm and in the Flow Diagram in Figure 4

<table>
<thead>
<tr>
<th>Cursor parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_s$</td>
<td>user-defined starting position of the cursor</td>
</tr>
<tr>
<td>$P_c$</td>
<td>previous position of the cursor</td>
</tr>
<tr>
<td>$P_n$</td>
<td>new position of the cursor</td>
</tr>
<tr>
<td>$d_i$</td>
<td>starting direction of the cursor</td>
</tr>
<tr>
<td>$d_n$</td>
<td>direction in which the cursor makes a new step</td>
</tr>
<tr>
<td>$h$</td>
<td>lateral grid size of the confocal image</td>
</tr>
<tr>
<td>$h_o$</td>
<td>angle defining the angular distance between successive &quot;rings&quot;</td>
</tr>
<tr>
<td>$v$</td>
<td>angle defining the angular distance between directions in each &quot;ring&quot;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Kernel parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$m_o$</td>
<td>width of the kernel (expressed in $h$)</td>
</tr>
<tr>
<td>$m_r$</td>
<td>length of the kernel (expressed in $h$) in the spherical kernel $m_r = m_o$</td>
</tr>
</tbody>
</table>

3. The accuracy of the algorithm is improved by allowing the cursor to step "in between voxels." Therefore, the positions within the image at which the light density is evaluated will most often not be on the image grid. The image voxel values are then trilinearly interpolated, taking into account the difference in lateral and axial resolution of a 3-D confocal image.

4. When $d_{e,0}$ (the best direction from the limited set of directions taken into consideration) is selected, the final direction in which a step is made, $d_n$, is calculated by interpolation of $d_{e,0}$ and its direct neighbours on the sphere-grid that is formed by the successive rings, and normalising the result to the lateral grid unit $h$:  

$$d_n = \text{norm}(g(\Phi, \Theta) \cdot d_{e,0} + g(\Phi, \Theta - h_o) \cdot d_{e-\Delta,0} + g(\Phi - h_o, \Theta) \cdot d_{e,\Delta,0} + g(\Phi + h_o, \Theta) \cdot d_{e,\Delta,0})$$

where $g(\Phi, \Theta)$ is the outcome of the convolution in the direction $d_{e,0}$.

The new position $P_n$ on the axis is then calculated as:  

$$P_n = P_s + h_o \cdot d_n$$

where $h_o$ is the stepsize.

After $P_n$ is stored, the entire procedure is repeated with $d_n = d_{e,0}$ and $P_s = P_n$. The algorithm stops when the new position, $P_n$, is close to the user-defined stop position $P_c$, $|P_n - P_c| < \epsilon$, where $\epsilon$ is best set in the range $3h < \epsilon < 6h$. Figure 4 shows a flow diagram of the entire tracing algorithm.

Computer-Generated Images

To establish accuracy and bias of the algorithm, computer-generated three-dimensional images of tori (a torus is a circular tube) with varying radii were traced. The tracing results were compared with the mathematically defined axes of the tori. The tori were generated by calculating the grey value for every voxel in the image as a Gaussian function of the distance $d$ to a circle $O(x,y,z)$ with radius $r_c$ centred in the middle of the image, that is, every voxel in the test image $I$ satisfies the condition $I(x,y,z) = A \cdot e^{-\left(d/R^2\right)}$, where $R$ determines the thickness of the torus, $d$ is the shortest distance to the circle, and $A = 150$. The tori all have $R = 4h$, and have curvature (expressed in $1/r_c$) of 1/8, 1/16, 1/32, and 1/48. Images with increasing artificial noise levels (of tori with curvature 1/32) are generated by randomly choosing a new voxel value from the Gaussian distribution based on the original voxel value: $N(f(x,y,z),\sigma)$.

Chromosome Images

Chromosome images of Crepis capillaris root tips were used to test the algorithm. C. capillaris plants were selected because of the low number (2n = 6) of easily recognisable chromosomes. A comprehensive description of the fixation and staining technique was given by Oud et al. (16), and is summarised as follows: 1) ethanol-acetic acid (3:1) fixed root tips were stained with a DNA-specific Feulgen staining procedure; 2) the cells were placed between two cover slips; care was taken to avoid squashing; 3) to excite the Feulgen fluorescence, the 530.9 nm line of a krypton ion laser was used in combination with a 580 nm dichroic mirror and a 580 nm blocking filter (for technical details, see ref. 4); 4) the prophase nucleus shown in the paper was scanned in varying numbers of sections with the confocal microscope developed in our department (3,4); each section consists of 256 x 256 pixels of 100 x 100 nm each, which gives an optimal lateral resolution in the confocal microscope that was used; the mutual distance between the optical sections was 400 nm; and 5) the raw fluorescence intensity data were stored as integers with a value between 0 and 255 in an 8 bit memory array as a function of the position in the preparation.

Tests with Computer-Generated Images

To establish the accuracy and bias of the algorithm and to optimise parameter settings, the artificial images described above were traced by the algorithm with various parameter settings. In each torus 50 points along the medial axis were traced. Each experiment was conducted with both kernel types. In all experiments the step size was equal to the lateral grid size; the angular increment $h_o$, defining the number of positions tested per ring, was $h_o = 1/4\pi$. The kernel length was set at the value of kernel width: $m_r = m_o$. No images with artificial noise were used in the first two experiments. The following three experiments were conducted:

1. $h_o$ was varied over .01, .02, .03, and .04 rad, with $m_r = 3h$
2. $m_r$ was varied from 2 to 5 times the grid size with optimal $h_o$
3. The images of the torus with radius 32 with in-
increasing artificial noise levels were traced with optimal parameter settings derived from experiments 1 and 2.

**Test with Real Chromosome Images**

As an example of the actual use of the cursor program and to demonstrate the performance in practice, six chromosomes of a selected prophase nucleus of *C. capillaris* (Figs. 1, 7) were traced. For each chromosome, the starting point and starting direction as well as the stopping point were indicated manually using the procedure described above under Description of the Cursor Program. The chromosomes were traced with both kernel types, with the optimal parameter settings derived from the experiments with computer-generated images. The resulting three-dimensional coordinate strings were used to generate an artificial three-dimensional image in the same way as the images of tori were generated. The light intensity values in the artificial image \( f_a \) depend on the shortest distance to the three-dimensional coordinate string in a Gaussian way:

\[
    f_a(x,y,z) = A e^{-d(R)^2},
\]

where \( R \) determines the thickness of the artificial chromosome, \( d \) is the shortest distance to the coordinate string, and \( A = 150 \). By visualisation of the artificial images the tracing result can be evaluated.
**Quality Assessment**

For each result of the experiments with computer-generated images, the bias $b$ of the algorithm was calculated as the difference between the mean distance of the points to the centre of the torus and the actual radius $r$:

$$ b = r - \frac{\sum_{i=1}^{n} d_i}{n} $$

As a measure for the accuracy of the algorithm the standard deviation $sd$ was then calculated using the distance $d_i$ of each of the recorded points to the circle with radius $r_f - b$:

$$ sd = \sqrt{\frac{\sum_{i=1}^{n} d_i^2}{n-1}} $$

**RESULTS**

**Computer-Generated Images**

Figures 5 and 6 show the data from the test experiments. In Figure 5a and b the bias of the algorithm is shown for four different values of the angular deviation $h_w$ for the spherical kernel type 1 (a) and for the tubular kernel type 2 (b). The standard deviation of the same data is plotted in Figure 5c and d. For both kernel types the bias decreases with decreasing $h_w$, changing from positive to negative below $h_w = 0.02$ rad. The $sd$ was also lowest for $h_w = 0.02$ rad. With values below $h_w = 0.02$ rad, the algorithm showed some instability. Therefore $h_w = 0.02$ was selected as the optimal value for the further experiments. In Figure 6 bias (a and b) and $sd$ (c and d) for both kernel types are plotted against curvature for different values of the size of the kernel $m_r$. The second type shows a greater positive bias than type 1, especially for the higher values of $m_r$. In contrast, the $sd$ does not differ very much for both kernel types, and is very low in general (in the worst case it is still less than 1 voxel, that is, 1 voxel). When artificial noise is added to the image, the bias and $sd$ increase somewhat (data not shown), but even in images with 40% noise level the $sd$ remains below 1 voxel (for both kernel types ~0.2 voxel).

**Real Chromosome Images**

Figure 7a shows three-dimensional artificial chromosome images as they were obtained with the cursor program. The images were visualised using the Simulated Fluorescence Process (SFP) method (20). The left image shows the chromosome that was traced first (Fig. 1). In each subsequent image, a chromosome is added. Figure 7b shows an SFP image of the original confocal image.

The chromosomes were traced with both kernel types. When kernel type 1 was used the cursor often kept circling around in areas with relatively high light intensity. With the use of kernel type 2 with sizes $m_r = 3$ and $m_z = 6$, this problem was successfully avoided. The chromosomes shown in Figure 7a are all based on the 3-D coordinate data obtained by tracing with kernel type 2.

**DISCUSSION**

In this paper an algorithm has been presented to automatically obtain the 3-D coordinate strings representing the medial axes of chromosomes (or other tubular structures) in a digitized 3-D image. The accuracy and bias of the algorithm has been established by tracing mathematically defined test images and comparing the resulting coordinate string with the mathematically defined path.

From the bias figures shown in the results, it can be seen that the algorithm generally has a positive bias, implying a tendency to short-cut the curves. This tendency increases with increasing curvature, although with optimal parameter settings it remains low. The
Fig. 5. Graphs of the bias (a and b) and standard deviation (c and d) plotted against curvature for four different values of $h_v$, with kernel type 1 (a and c) and 2 (b and d). The value of the kernel size parameters was $m_r = m_t = 3h$, and the step size was $h_s = 1h$.

Fig. 6. Graphs of the bias (a and b) and standard deviation (c and d) plotted against curvature for four different values of $m_r$, with kernel type 1 (a and c) and 2 (b and d). $h_v$ was set at the optimal value $h_v = 0.02$ rad, and the step size was $h_s = 1h$. 

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FIG. 7. a: Computer-generated three-dimensional image of the nucleus shown in Figure 1 based on the three-dimensional coordinate strings obtained with the cursor. The top left picture shows the first chromosome that was traced (see Fig. 1c). In each successive picture a chromosome is added. The three-dimensional images were generated in a way similar to the three-dimensional test images of the tori (see Materials and Methods): the light intensity values in the image depend in a Gaussian way on the shortest distance to the three-dimensional coordinate string obtained by the cursor. The images are visualized by the so-called Simulated Fluorescence Process method (SFP) described by van der Voort et al. (20). b: SFP image of the original three-dimensional data set also shown in Figure 1.

results of tracing real chromosomes can be corrected for the bias by calibration based on a curve, as shown in Figures 5 and 6 for the specific parameter settings. Applying such a bias correction yields an accuracy which is the same for both kernel types. With the optimal parameter settings the standard deviation is less than 10% of the grid size (0.1 μm). In a 3-D CSLM image with optimal lateral and axial resolution [100 and 400 nm, respectively (3)], this means a lateral deviation of 10 nm and an axial deviation of 40 nm. When noise is added to the data, the sd remains far below 1μm. Therefore it can be concluded that the error caused by the inaccuracy and bias of the algorithm is negligible compared with errors introduced by the quality of real chromosome images.

The robustness of the algorithm was tested with real confocal chromosome images. Three types of problems may occur:

1. When a volume of relatively high light intensity values within the chromosome is encountered the cursor will be attracted to it. In the worst case it gets trapped, and will keep circling around in this volume. This problem is successfully avoided in many cases by the use of kernel type 2, with a high value of \( m_h \), the length of the kernel if necessary. The reason kernel type 2 is less sensitive to this type of aberration in the image is clarified in Figure 3: because of the distribution of its weight factors, kernel type 2 will not easily deviate to incidental spots of higher light intensities from a path of successive, lower, but still relatively high, light intensities.

2. When two chromosomes are locally too close to one another, the cursor will follow the path with highest light intensities, which is not necessarily the correct branch. The problem is dealt with by a restricted manual intervention, locally removing the chromosome with highest light intensity values from the image with the aid of a procedure we described elsewhere (9). The other chromosome can then first be pursued.

3. Starting and stopping points are indicated manually. Errors introduced by the user could corrupt the comparison of different chromosome representations. It is therefore desirable that some objective criterion be provided to determine these positions automatically. In the case of chromosomes it is possible to perform an additional staining, in which the telomeres (endings) of chromosomes are specifically stained by in situ hybridization of telomere-specific DNA sequences. The centre of these telomeres can then be detected in a way similar to location of the medial axis. In complex chromosome images of early prophases, in which chromosomes cannot be traced over the whole length, in situ hybridization of chromosome-specific sequences provides im-
portant information on where to continue after stop-

From the data presented it can be concluded that the
cursor algorithm provides for an objective and there-
fore reproducible method to trace automatically the
medial axes of prophase chromosomes. In comparison
with algorithms such as grey distance transform or
skeletising, the cursor algorithm has the advantage
that the output is a string of ordered 3-D coordinates,
whereas the other algorithms' outputs are still 3-D im-
ages that have to be processed (interactively or auto-
matically) in order to obtain ordered 3-D coordinates
along the axis. Finally, this method could allow a more
extensive understanding of the (changes in) chromo-
some organisation throughout the cell cycle, its rela-
tion to cell function, and the complex process of chro-
mosome condensation.

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