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VISUALIZATION AND ANALYSIS TECHNIQUES FOR THREE DIMENSIONAL INFORMATION ACQUIRED BY CONFOCAL MICROSCOPY

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Abstract

Confocal Scanning Laser Microscopy (CSLM) is particularly well suited for the acquisition of 3-dimensional data of microscopic objects. In the CSLM a specific volume in the object is sampled during the imaging process and the result is stored in a digital computer as a three-dimensional memory array. Optimal use of these data requires both the development of effective visual representations as well as analysis methods. In addition to the well known stereoscopic representation method a number of alternatives for various purposes are presented. When rendering in terms of solid-looking or semitransparent objects is required, an algorithm based on a simulated process of excitation and fluorescence is very suitable. Graphic techniques can be used to examine the 3-dimensional shape of surfaces. For (near-)real time applications a representation method should not require extensive previous data-processing or analysis.

From the very extensive field of 3-D image analysis two examples are given.

Key words: 3-dimensional confocal, light microscopy, fluorescence, laser microscopy, 3-D image processing, 3-D image analysis, graphics, solid model, stereoscopic imaging.

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Introduction

For the study of the 3-dimensional morphology of biological objects confocal microscopy has been demonstrated to be a very powerful technique (Brakenhoff et al., 1979, 1985, 1988; Wijnaendts van Resandt et al., 1985; Carlsson et al., 1985). The confocal microscope can easily and rapidly collect information over the volume of a 3-dimensional specimen without the need of extensive specimen preparation and mechanical sectioning, as in electron microscopy.

The key is that in confocal microscopy we are able to sample just one volume element in a specimen, in first approximation, independently of its surroundings. An image is then created by scanning this sample element through the volume of the specimen. When coupled to a computer system the data can be directly stored in a 3-dimensional computer memory array, from which the desired image, etc., can be generated later. Before discussing the main subject of this paper we will summarize a few properties of this type of imaging. In comparison with conventional light microscopy the lateral resolution is improved (for a point object by a factor of 1.38), but the main difference is that the optical arrangement in confocal microscopy gives us the optical sectioning property: only radiation from a chosen image plane will contribute to the image while light from the off-focus layers is effectively suppressed. In conventional microscopy these off-focus contributions lead to a strong loss of image contrast. The confocal microscope is most successfully used in fluorescence, while reflection and transmission operations modes are also possible. For further details see Brakenhoff et al. (1979), van der Voort et al. (1985), Wilson and Sheppard (1984).

A typical size of the sampled volume element in fluorescence (excitation wavelength 480 nm, detection above 520 nm) is 200 nm x 200 nm x 800 nm when high numerical aperture immersion optics (N.A. = 1.3) are used. For scanning, various strategies can be followed; either specimen scanning, beam scanning or by the Nipkov disk method (for details see Brakenhoff et al., 1988). The image collection times with typical laser light sources of 20-100 mW power, tend to be independent of the method, as for a desired signal to noise ratio in the image, a certain number of photons have to be collected for each data point. Only at very

intense sources the data collection rate in mechanical scanning may be limiting. For a typical 3-D 16 section 256 x 256 data set we collect the data with 8 bit precision in about 80 seconds. Elements in the 3-D data set are called voxels. Below we demonstrate a few basic ways to represent these 3-D data followed by two examples of how these data can be analysed in order to reach certain biological conclusions. Where relevant we also indicated some filtering and image-processing operations which have been employed to generate the images, but we will not treat this aspect in detail.

Sections and cross sections

A straightforward and very informative representation mode of 3-D data sets is of a series of sections acquired at increasing depth. An example is given in Fig. 1a and we will use this data set to illustrate some aspects of 3-D imaging. It often occurs, especially during 3-D confocal microscopy of semi-transparent materials, that the image intensity received from deeper lying sections becomes progressively lower. This is due to the absorption not only of the illuminating light on its way to the imaged section but also of the generated fluorescence light on its way back to the collecting lens. This happens while the sectioning effect is operational and good image quality is preserved.

A first order correction of this effect can be realized by multiplying the detected intensity from each layer by an empirical determined quantity such that the apparent intensity from each layer is about equal. This procedure works well when scattering/absorption takes place approximately uniformly over the volume. Fig. 1b shows the result of such an operation where the deeper lying sections have been multiplied by a proportionally increasing factor such that the deepest layer was multiplied by a factor 2.2.

The depth information in the specimen can be presented as a number of x - z cross sections, where the z-axis is along the optical axis and x and y are the lateral coordinates (Fig. 2) Series of cross sections like these can provide information over the exact depth positioning of specific structures.

Stereoscopy and superposition images

The method for the construction of stereoscopic image pairs from 3-D data sets has been published before. (van der Voort et al., 1985; Carlsson et al., 1985; Brakenhoff et al., 1988). In summary, one constructs one image of such a pair in the computer by projecting the 3-D data set on the image plane along a certain projection direction. With each pixel in the image-to-be corresponds a projection line through the data set. To determine the value to be assigned to that pixel from the voxel values encountered along the projection line, one can use various algorithms such as additions, retaining the maximum, or retaining the minimum. Two images with different projection directions form a stereo pair. One such image we will call a superposition image and may be useful to get an impression of the contents of a 3-D data set. As an example, we show in Fig. 3 a

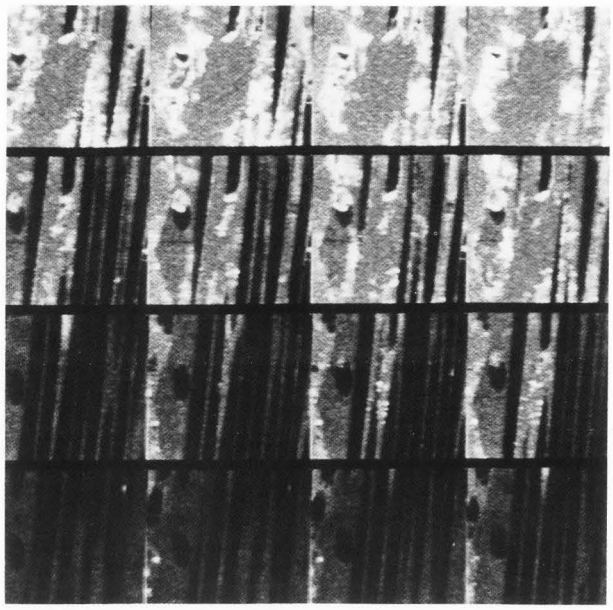


Fig. 1a. 16 Optical sections of a piece of material consisting of transparent fibers embedded in a fluorescent matrix, as acquired by confocal microscopy operating in fluorescence. The sections were taken starting from above the material - top right image - going down into the material up to the deepest lying sections - bottom left image. The mutual sections distance is 1.5 μm. Bar = 75 μm.

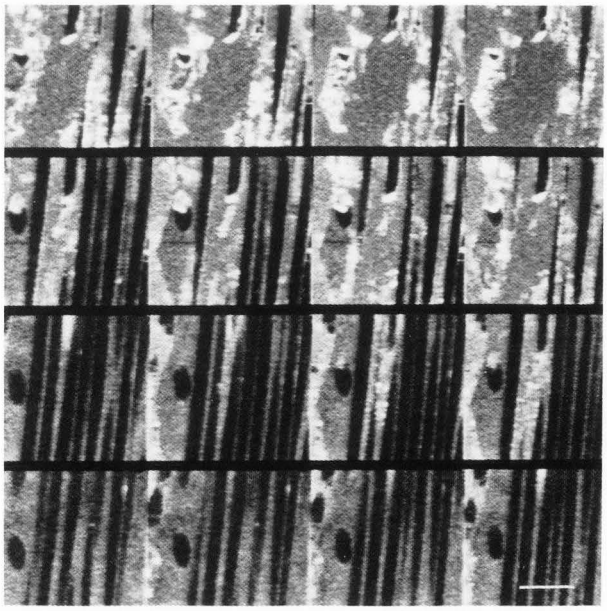


Fig. 1b. The data set of Fig. 1a after correction (see text).

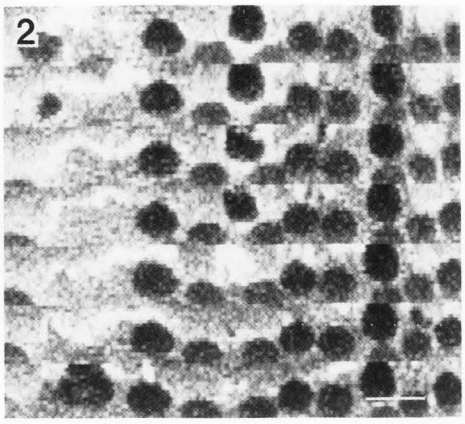


Fig. 2 (above). A series of 7 cross sections through the data set displayed in Fig. 1b. The cross sections are made at distances equally spaced along the y-axis. The dark circular patches are the vertical cross sections through the non-fluorescing fiber. Bar = 25 μm.

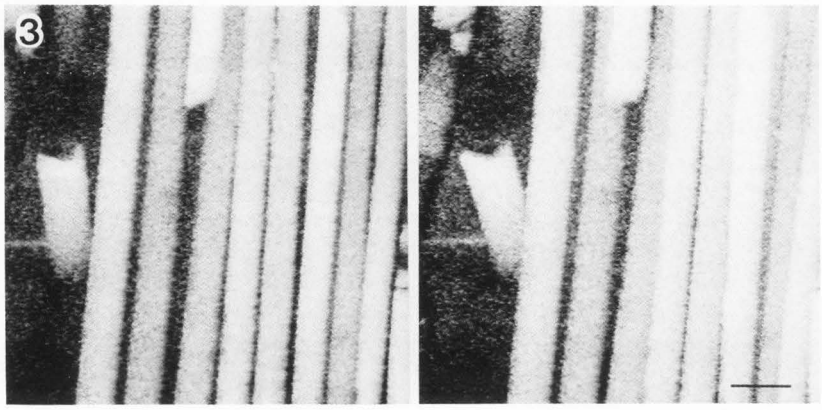


Fig. 3 (right). Stereoscopic image of the fiber structure contained in the data of Fig. 1b. In the generating algorithm the minimum along the projection lines has been retained and the resulting stereoscopic images have been contrast inverted. See text. Observation tilt angle 0.25 radian. Bar = 25 μm.

stereoscopic image pair of the fiber arrangement present in the data set of Fig. 1b. We retained the minimum along the projection lines and to facilitate the viewing of the non-fluorescent fibers we inverted the contrast. Thus, thanks to appropriate processing steps one can visualize non-fluorescing structures in a fluorescent medium equally well as directly fluorescing structures.

An alternative way of presenting stereoscopic images is to present these images as stereoscopic image arrays. These are constructed, as described above; along projection lines tilted at regularly spaced angles with respect to both the x and y plane. Fig. 4 shows an example of a 3 x 3 stereoscopic array. The virtue of this representation is that each pair of images viewed in any orientation gives a stereoscopic view. Being able to change in such array one's viewing point at will is very useful during the examination of complicated structures, as often certain structural details, for instance deep in the data set, are only visible because of obscuration from specific angles. We constructed stereoscopic arrays of 4 x 4 and 5 x 5 images and with some experience rapid, stereoscopic inspection from various viewpoints is quite easy. For the algorithm to be used along the viewing line more complicated possibilities can be chosen, like maximum positive gradient, etc., but the success of the Simulated Fluorescence Process described below makes it for the time being less relevant to investigate these alternatives.

Spatial representation by the Simulated Fluorescence Process (SFP) method

The SFP method is a computer simulation consisting of two independent steps corresponding to the excitation and emission phase in a real fluorescence process. The starting point is the measured three dimensional voxel data set. This data set is "illuminated" from an excitation light source which is at present placed at infinity (parallel projection). Then in the first excitation step, when an exciting ray on its path through the data set encounters a non-zero voxel, the developed SFP algorithm will set the value of this voxel point equal to the triple product of the excitation intensity times the original voxel value in the 3-D image times the absorption factor. Also at this voxel point the intensity of the exciting light ray will be reduced due to absorption by a factor determined by the original voxel value times the absorption factor. Absorption at higher levels will thus affect the excitation of voxels deeper in the data set.

The second step in the SFP algorithm is the emission phase, where the voxels are assumed to radiate light in a certain direction, which may be different from the illumination direction. The amount of light an observer - again placed at infinity - receives from each voxel will again, as in the excitation phase, be affected by the intervening voxels. A background for the structure is created by adding below the deepest layer of the 3-D data a layer of voxels with a uniform intermediate value before starting the SFP process. The result of this operation as demonstrated in Fig. 5 and Fig. 6 is a very vivid representation of the 3-D structure of an object, thanks to the natural way structural elements are highlighted and shadows thrown. The apparent transparency of the objects represented can be influenced by the choice of absorption factors used in the excitation and emission phase of the process. See for further details van der Voort et al. (1988).

A practical advantage SFP method over the stereoscopic image representations is that some people are not able to perceive depth from stereoscopic images (Richards, 1970). Also observers of stereoscopic images do sometimes not

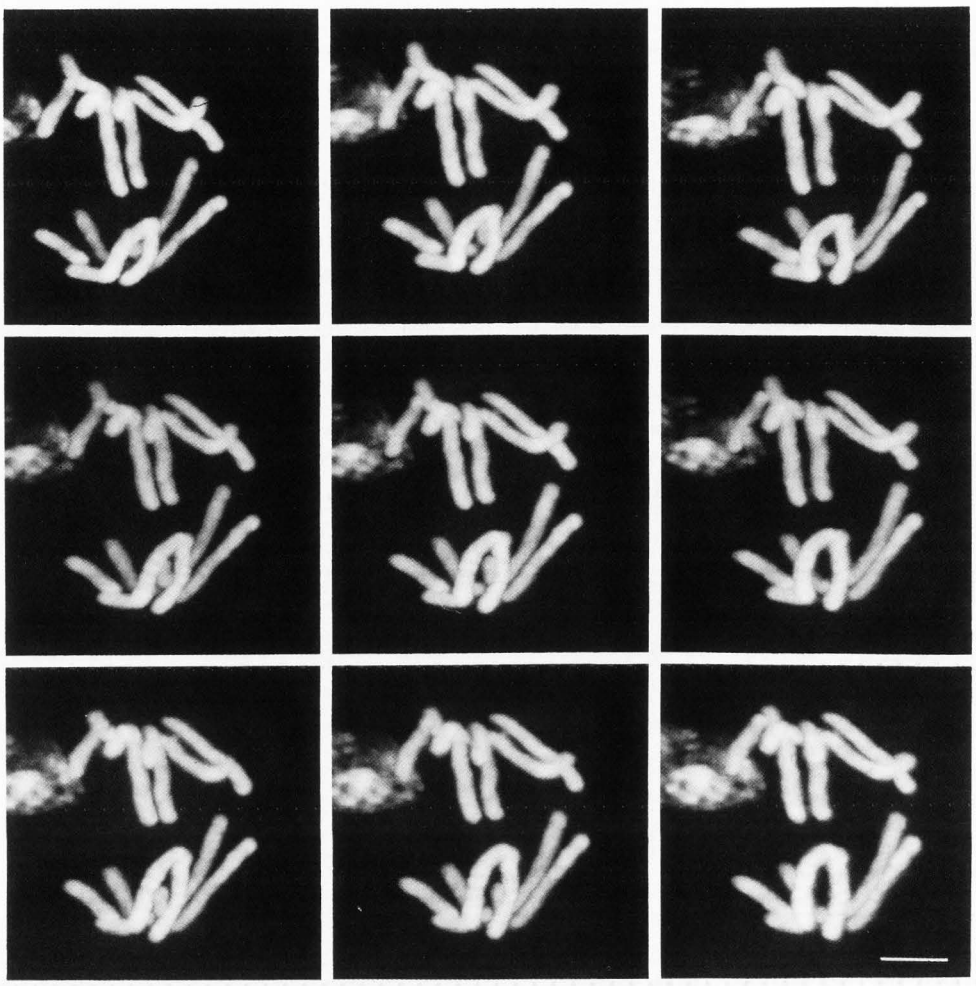


Fig. 4. Stereoscopic 3 x 3 array of the chromosome structure in a metaphase nucleus of Crepis capillaris. Each two images in any orientation provide a stereoscopic view. The central image is the perpendicular view, while the others are observed under tilt angle of ± 0.1 radian either in the x direction or y direction or both for the corner views. Bar = 3.6 µm.

easily agree on the relative depth relations in the images.

The analysis of chromosome arrangements

The necessity of confocal microscopy for the analysis of complicated 3-D biological structures, is well demonstrated in the study of the spatial ordering of chromosomes in Crepis capillaris (Smooth Hawksbeard).

For more than a century, there has been a discussion about the question whether chromosomes show a random distribution with respect to each other or not. For example, an association of homologous chromosomes is reported in somatic cells of some species, but is absent in others (see review by Avivi & Feldman, 1980). Most studies are carried out on squashed or air-dried preparations. Since in these preparations the cells are flattened on the objective glass, all interpretations about the spatial ordering of chromosomes are made from 2-D material. Another approach is the analysis of 3-D reconstructions from a large series of electron-microscopic sections. This method can only be done by a very experienced technician, and is extremely time consuming (Bennett, 1982; Heslop-Harrison et al., 1988). As a result the analysis of chromosome disposition has to be carried out with a limited number of cells, which restricts the possibility of drawing statistically satisfactory conclusions (Callow, 1985).

Confocal fluorescence microscopy, however, has proven to be the most suitable method to study chromosome arrangements in 3-D images (Oud et al., 1989). With the optical section technique it takes only a few minutes to make a 3-D image of a cell, which, for the first time, allows qualitative as well as quantitative studies. Moreover, the analysis can be carried out on the same type of preparations used for fluorescence light microscopy. The only difference is that squashing has to be omitted to preserve the third dimension.

For a quantitative study of the spatial ordering of chromosomes,we used the higher plant

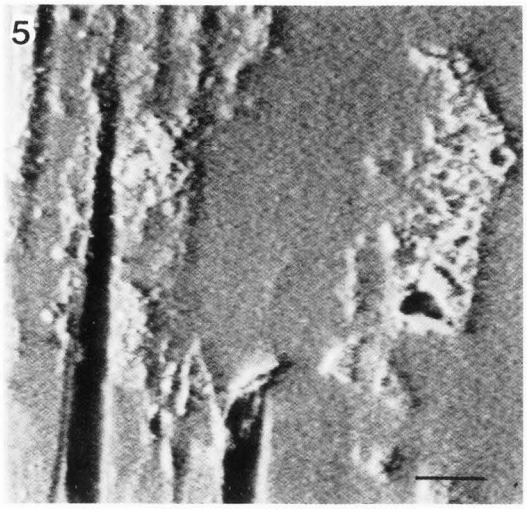


Fig. 5. S.F.P. image of the data set of Fig. 1b. With this representation method the features are displayed present at the surface along which the material was cut mechanically. Bar = 25 µm.

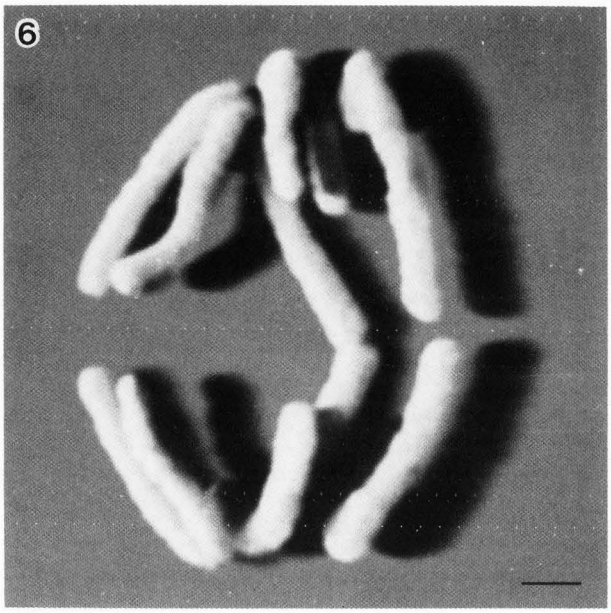


Fig. 6. S.F.P. image of a Crepis capilaris root tip anaphase 1-3: chromosome numbers. The chromatids of chromosome nr. 2 occupy adjacent positions; the numbers 1 and 3 occur in alternating order (arrangement D in fig. 7). The original 3-D image is made from 14 optical sections with mutual distance of approx. 850 nm. Bar = 1.6 µm.

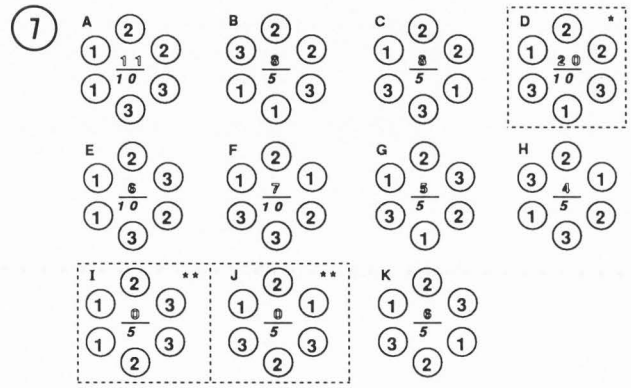


Fig. 7. Survey of the 11 chromosome arrangements (A-K), represented by their centromeres, as can be expected in Crepis capillaris. 1, 2, and 3 = centromeres with corresponding chromosome number. In the centre of each configuration the observed number of cells is printed in outline and the expected number of cells, assuming a random distribution, is printed with italics. The chi-square value for all 11 arrangements = 26.6 (p = 0.003). The most striking deviations from the expectation concern the excess of arrangement D (chi-square = 10.0, p = 0.002) and the absence of arrangements I and J (chi-square = 5.00, p = 0.025).

Crepis capillaris with 2n=6 chromosomes, which can easily be identified. In dividing root tip cells of this species, the centromeres are circularly arranged. This phenomenon and the impossibility to distinguish between maternally and paternally derived chromosomes, restricts the number of different chromosome arrangements to eleven configurations. All other arrangements are mirror images of these eleven. For a statistical analysis with the chi-square test, an expected number of at least 5 cells for each of the arrangements is necessary. If we assume a random distribution, 75 cells have to be analysed, in which four arrangements are expected twice as often as the remaining seven.

Root tip cells are stained with a DNA specific (pararosaniline or mithramycine) fluorochrome. The analysis has been carried out on cells in the anaphase stage of mitosis. Of each anaphase a stereo pair of 3-D images has been made using the maximalization algorithm on the basis of 10 to 20 median filtered optical sections. These stereo images are very useful for a quick analysis of the chromosome positions. SFP images are an elegant way of reproducing 3-D images at the 2-D level (fig. 6). Sometimes large chromosomes at the front hide other chromosomes. In those cases it is necessary to revert the order of the sections and to make a second SFP image from the back side.

Fig. 7 shows the data of the analysis of 75 anaphases. The main results are: (a) there is a clear preference for an association of the chromosomes 2, (with a nucleolus organizer region or NOR; carrying the rRNA genes), (b) there is a significant surplus of cells with an arrangement in which the NOR chromosomes are juxtaposed and the other two pairs of chromosomes occur in alternating order (fig. 6), and (c) two of the three arrangements with the NOR chromosomes in opposite direction are never observed in this material.

Spatial distribution of centromers in interphase nuclei

Over the past decades a wealth of information on gene structure and the control of gene expression via nearby regulatory elements has become available. In contrast, we know very little about the way a cell coordinates gene expression, e.g., during differentiation. We and others postulate that the spatial organization of the cell nucleus is instrumental in higher order control of gene expression (Nelson et al., 1986). Determination of the 3-D distribution of chromosomes in the nucleus is one approach to obtain insight in the statics and dynamics of nuclear organization. To this end we are analysing the spatial distribution of chromosome centromeres in human fibroblasts by confocal microscopy.

Centromeres were visualized after indirect immunofluorescent staining using automo-immune sera. Fig. 8a shows a superposition of 16 optical sections (spaced 0.65 µm along the optical axis) of a 3-D confocal image of a human fibroblast nucleus. The centromeres are the brightly fluorescent spots against the weakly fluorescent background. For the identification of the centromeres within the 3-D image of the nucleus, we used the following procedure: As a first step we applied a Sobel filter to the 3-D data set. (Gonzalez and Wintz, 1987) This filter determines around each voxel the strength and direction of the gradient of fluorescent intensity.

For a well-defined, individual centromere we find after Sobel filtering a shell-like structure with a radius that is equal to the distance of the maximum gradient value to the centromere centre. To determine the position of less well-separated centromeres we used a template matching algorithm which compares, over the whole data space, the ideal shell configuration with the gradient of the intensity distribu-tion as determined by the Sobel filter. As can be seen from the super-position image of the centromere locations found by this procedure (Fig. 8b), this approach is quite effective. Evidently, centromeres that are located in close proximity are recognized well.

In 3-D images of the nuclei of these fibroblasts we counted 40 to 45 centromere spots. Considering the fact that not all centromeres are stained with equal intensity and that centromere pairing may occur that cannot be resolved by our routines, this number agrees well with the known number of chromosomes in a human cell,i.e., 46. By integrating over the volume each fluorescent centromere structure we can determine the total fluorescence of each individual centromere. Fig. 8c shows a histogram of the frequency of occurrence of total fluorescence per centromere in the nucleus. We are presently involved in statistically analysing the 3-D spatial distribution of centromeres in a population of identical fibroblast cells. Results will give important insight in the plasticity of nuclear organization, i.e., the cell to cell variation in 3-D distribution.

Comments and conclusions

Above we concentrated on representation methods which can be implemented efficiently in a computer system. This opens up the possibility of generating stereoscopic and SFP images and of course the sections/ cross sections within seconds after collection of 3-D data. This is important in a user environment, as one wants to be able to judge immediately whether a certain observed biological 3-D structure is significant and should be stored, or perhaps replaced by another 3-D image. Processing afterwards means that large quantities of digital data have to be manipulated, of which only a part is useful. The effectiveness of the stereoscopic and SFP algorithms is mainly due to the fact that the basic data, as collected by the microscope, are used without preprocessing. This is not the case when graphics techniques are employed, which require an analytic description of the object. In most cases this description consists of a specification of the boundary surface of the object. (Rogers and Adams, 1976). For this there exist various approaches (Tuohy et al, 1987, Coggins et al., 1986) but they have in common that they require a large number of calculations. The reward, however, is that these boundary data can be used by a graphics package running on a suitable work station. Then at high speed, often interactive, one can view the object from any point of view as desired. This makes graphic techniques more suitable for the post processing stage. With respect to image analysis of 3-D images we have given two examples to indicate just some of the possibilities. We expect that subsequent developments will take place especially in this area, thus realizing the true potential of confocal microscopy for the spatial analysis of biological and other structures.

References

Avivi L, Feldman M (1980) Arrangement of chromosomes in the inter-phase nucleus of plants. Hum Genet. 55: 281-295.

Bennett MD (1982) Nucleotypic basis of the spatial ordering of chromosomes on eukaryotes and the implications of the order for genome evolution and phenotypic variation. In: Genome evolution. Dover GA, Flavell RB, (eds), Acad. Press, London. 239-261.

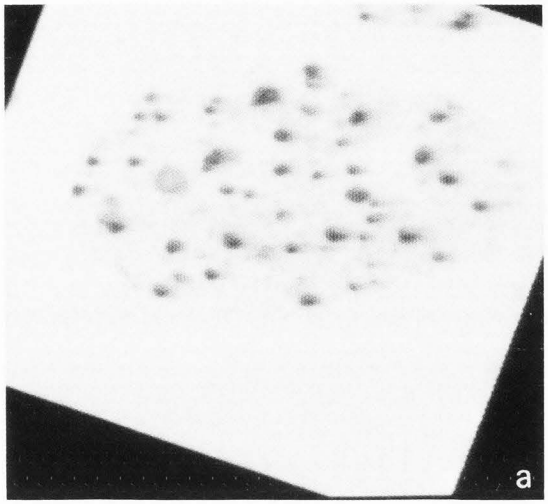
Brakenhoff GJ, Blom P, Barends P (1979) Confocal scanning light microscopy with high aperture immersion lenses. J. Microsc. 117: 219-232.

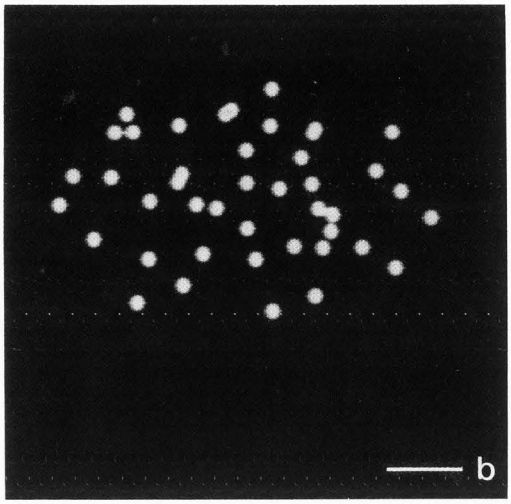
Brakenhoff GJ, Van der Voort HTM, Van Spronsen EA, Nanninga N. (1988). 3-Dimensional imaging of biological structures by high resolution confocal scanning laser microscopy. Scanning Microsc. 2: 33-40.

Brakenhoff GJ, van der Voort HTM, van Spronsen EA, Linnemans WAM, Nanninga N. (1985) Three-dimensional distribution in neuroblastoma nuclei shown by confocal scanning laser microscopy. Nature 317: 748-749.

Callow RS (1985) Comments on Bennett's model of somatic chromosome dispostion. Heredity 54: 171-177.

Carlsson K, Danielson PE, Lenz R, Liljeborg A, Maglöf L, Ashund N (1985) Three dimensional microscopy using a confocal scanning laser





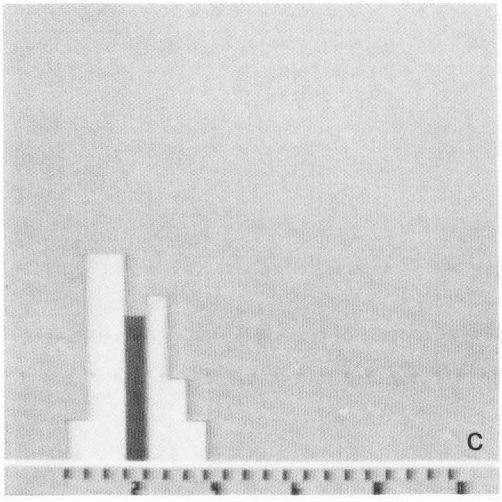


Fig. 8a. Superposition image of 16 sections of the nucleus of a human fibroblast cell with centromeres (bright spots) as acquired by confocal microscopy. Bar = 3.75 µm.

Fig. 8b. Superposition image of locations of centromeres as found by the template matching method.

Fig. 8c. Histogram of the fluorescent label distributions of the centromeres of the nucleus of Fig. 8a. Horizontal: fluorescence label per centromere, vertical: frequency of occurrence.

microscope. Opt. Lett. 10: 53-55.

Coggins JM, Fay FS, Fogarty KE (1986) Development and applications of a three-dimensional artificial visual system. Computer Methods and Programs on Biomed. 22: 69-77.

Gonzalez RC, Wintz P (1987) Digital Image processing Addison-Wesley Publishing Company, Reading, Ma. USA.

Heslop-Harrison JS, Smith JB, Bennett MD (1988) The absence of the somatic association of centromeres of homologous chromosomes in grass mitotic methaphases. Chromosoma (Berl.) 96: 119-131.

Nelson WG, Pienta KJ, Barrach ER, Coffey DS (1986) The role of the nuclear matrix in the organization and function of DNA. Ann. Rev. Biophys. Biophys. Chem. 15: 457-475.

Oud JL, Brakenhoff GJ, Van der Voort HTM, Van Spronsen EA, Nanninga N (1989) 3-D chromosome arrangement of Crepis capillaris in mitotic prophase and anaphase as studied by confocal scanning laser microscopy. J. of Cell Science (accepted for publication).

Richards W (1970) Stereopsis and Stereoblindness. Exp. Brain Res. 10: 380-388.

Rogers DF, Adams JA (1976) Mathematical elements for computer graphics. McGraw-Hill NY, NY, USA.

Tuohy M, McConchie C, Knox RB, Szarski L, Askin A (1987) Computer assisted three-dimensional reconstruction technology in plant cell image analysis, applications of interactive computer graphics. J. Microsc. 147: 83-88.

Van der Voort HTM, Brakenhoff GJ, Valkenburg JAC, Nanninga N (1985) Design and use of a computer-controlled confocal microscope. Scanning 7: 66-78.

Van der Voort HTM, Brakenhoff GJ, Baarslag MW (1988) Three-Dimensional Visualisation Methods for Confocal Microscopy. J. of Microsc. (1988, in press)

Wijnaendts van Resandt RW, Marsman HJB, Kaplan R, Davoust J, Steltzer EHK, Sticker R. (1985) Optical fluorescence microscopy in 3 dimensions: microtomoscopy. J. of Microsc. 138: 29-34.

Wilson T, Sheppard CJR (1984) Theory and Practice of Scanning Optical Microscopy. Academic Press London.

Discussion with Reviewers

R.W. Wijnaendts van Resandt: From studies of single plant chromosomes to human fibroblasts is a very large step. What is the real goal of this project?
Authors: The authors are very much aware of the difference in complexity between this simple plant chromosome and the genetic structures in other organisms. The goal of the project at this moment is to show that three-dimensional information, especially with appropriate image processing and representation techniques, opens up new possibilities for description and identification of 3-D structures, among which are chromosomes.

R.W. Wijnaendts van Resandt: Image analysis of 3-D images with the help of a graphic package is discussed in the comments and conclusions section. It seems to me that graphic systems are limited to display techniques and perhaps geometric analysis. How do you think graphic processing is able to extract quantitative data from 3-D micrographs?
Authors: In general we agree, may be there is a limited role for processing on the graphic level for the extraction of certain quantitative data like volume and surface area and measurement of curvature.

Reviewer III: How would the image of Figure 1(b) differ if you had merely chosen an area on each image of Figure 1(a) and simply normalized all the images such that this region was of the same average brightness in all cases?
Authors: This does not work since the same area in each image does not exist. The 2-D images come from different depths in the specimen and do therefore image a different part of the specimen every time. Only a general criterion operating over the whole image can be used.

Reviewer III: Could you give an indication of how many slices you use and the angle, or amount of offset, that you give to the sections to produce your stereopairs and stereoscopic arrays?
Authors: We collect standard data from 16 slices and use these for our stereoscopic images. The object used for the construction of Fig. 3 is 1 pixel point from slice to slice. This results in a reviewing angle of 29 degrees.