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CONFOCAL SCANNING LASER MICROSCOPY: FROM 3-D DATA COLLECTION TO 3-D IMAGE VISUALIZATION AND ANALYSIS

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Abstract. Confocal Scanning Laser Microscopy (CSLM) is particularly suitable for collection of data over the *volume* of biological specimen. This capability is based on its optical sectioning property. When coupled to a computer system one can create a direct 3-D representation of the object in computer memory. The optimal use of these data requires the development of effective visual presentation and analysis methods.

The application of the above techniques is illustrated on an object of biological origin.

Confocal scanning microscopy is beginning to take its place among the recognized techniques for the study of the spatial organization of biological structures. As far as resolution is concerned it forms a bridge between the limited resolving capability of conventional light microscopy and the superior one of electron microscopy. However with respect to the latter technique confocal microscopy has two great advantages. First biological objects can be studied in their natural, watery environment—even living, as shown in this paper—, thus avoiding the negative influence on morphology of the preparation techniques necessary for electron microscopy. These are among other chemical fixation, dehydration, embedding and sectioning. Volume shrinkages of up to 50% have, for instance, been observed in bacteria as a consequence of such preparation steps (Woldringh *et al.*, 1976). Second, confocal microscopy is capable of studying the structure over the volume of the material, while leaving the specimen basically intact. While in electron microscopy ultramicrotomy techniques have to be employed, in the confocal technique the depth information can be directly obtained, due to its optical sectioning.

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The idea of applying the confocal concept to light microscopy dates from the late 1970s. It was shown by Sheppard *et al.* (1977) that in confocal scanning microscopy a fundamental improvement in imaging, as compared to normal microscopy could be expected. Our group (Brakenhoff, 1979) showed that this expectation could indeed be realized in practice at the high numerical apertures (NA), where the resolution gain is of real practical value. Also the optical sectioning effect in the situation of diffraction-limited imaging was then demonstrated (Brakenhoff *et al.*, 1980). Earlier optical sectioning was demonstrated in the range governed by geometrical optics by Egger *et al.* (1967).

THE CONFOCAL MICROSCOPY TECHNIQUE

The basic principle of confocal microscopy is explained in Fig. 1. The arrangement shown is for operation in fluorescence. The principle can also be applied in transmission. For the theory of image formation we refer to Brakenhoff, 1979 and Wilson, 1984. Suffice to say here that the imaging can be considered to be determined by the interaction of the object with the product of the illumination

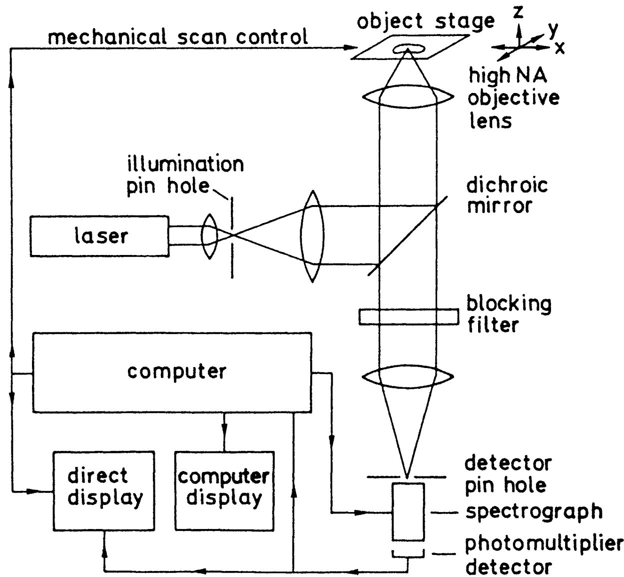


Fig. 1. Principle of the confocal scanning fluorescence microscope. Light originating from a laser-illuminated pinhole is focussed on a certain point of the object while the same point is also precisely imaged on a detector pinhole. The term confocal relates to the fact that the image of the illumination pinhole and the backprojection of the detection pinhole have a common focus in the object. The specimen is scanned mechanically through this confocal point and the image data, in addition to being displayed directly, are digitized and stored in a computer system. The optical arrangement dichroic beam splitter/blocking filter is as commonly used in fluorescence microscopy. The spectrograph enables one to select a certain band from the fluorescence radiation for image formation. Both the band width as well as the wavelength on the spectrograph can be set by the computer system. For confocal reflection microscopy the blocking filter is omitted and the dichroic mirror is replaced by a beamsplitter.

distribution and the detection sensitivity distribution. If these three-dimensional distributions are diffraction limited and overlap optimally it can be shown that improved imaging results in comparison with conventional microscopy (Sheppard *et al.*, 1977, Wilson *et al.*, 1984). For point objects for instance the lateral and axial resolution improve by a factor of 1.38. In order to obtain an absolute gain with respect to non-confocal microscopy, optics have to be used with maximum numerical aperture i.e. with N.A.=1.3 to 1.4. With good optics the expected improved confocal imaging can be shown to be realisable in practice (Brakenhoff *et al.*, 1979, 1980) resulting in observed point resolutions down to 130 to 140 nm.

The sectioning property in confocal microscopy deserves special attention. In normal microscopy all the radiation generated at various levels in the specimen will reach the image plane. This causes often a strong reduction in the contrast of the image of the in-focus level of the specimen. However in confocal microscopy the optical arrangement results in almost complete suppression of the contributions from the out-of-focus levels in the specimen. The sectioning power of confocal microscopy can perhaps best be realized from the fact that in normal fluorescence microscopy one cannot find the vertical position of a uniformly fluorescing layer in a specimen, while in confocal microscopy its height can be determined with the precision as defined by the improved axial response function.

In confocal microscopy one can either scan the beam (Wijnaendts van Resandt *et al.*, 1985, Carlsson *et al.*, 1985) or scan the specimen (Brakenhoff *et al.*, 1979, 1985) to obtain the specimen data. As indicated in Fig. 1 we use an instrument of the latter type. It is completely controlled by a computer system which takes care both of the instrument operation as well as the 3-D data collection, representation and processing functions presently available in the microscope.

The 3-D image acquisition is done with the help of a software routine automatically collecting the data from a series of 2-D images at various heights and storing these in memory. Such a 3-D data set we call a 3-D image. We collect typically data of 16 sections on a 256×256 pixel grid for each section. The collection of such a 1 Mbyte 3-D image takes typically 1 minute. For processing these data we have available the usual filter image processing routines, adapted for 3-D data sets. For 3-D image representation algorithms were developed for representation through stereoscopy and a Simulated Fluorescence Process (S.F.P.). In the first algorithm stereoscopic images are generated by making projections of the 3-D image along different directions (viewing lines). A pair of 2 such images with a not too large angle between viewing lines gives a satisfactory stereoscopic image. In the S.F.P. algorithm we simulate the fluorescence process by illuminating the 3-D data set in the computer from a certain angle. The 3-D image elements absorb in the simulation this radiation in proportion to their value and emit subsequently in proportion to the absorbed quantity. While passing a 3-D image element the radiation is absorbed in proportion to the element value, both in the absorbtion and re-emission fase. For further details on the system and the algorithms we refer to Van der Voort (1985, 1988).

CONFOCAL IMAGING EXAMPLES

The improved imaging characteristics of confocal imaging at high N.A. have been reported before. In fluorescence lateral and axial point resolutions at N.A. = 1.3 were observed of 220 and 800 nm respectively (Brakenhoff *et al.*, 1985).

We present CSLM pictures of living cells of the budding yeast *Saccharomyces cerevisiae* in which the mitochondria are stained by means of the fluorescent dye DASPMI (Bereiter-Hahn, 1976, Bereiter-Hahn *et al.*, 1983). An added reason for studying this object is that considerable controversy surrounds the number and structure of mitochondria in yeast cells as viewed in electron microscopy (cf. Hoffmann and Avers, 1973; Grimes *et al.*, 1974; Stevens, 1977; Tanaka and Kanbe, 1985).

We found a network-like organization of mitochondria in fast growing cells (see Figs. 2, 3, 4), however, these structures disappear and are replaced by the more traditional image of small ellipsoid shapes, when the cells are grown under conditions slow growth. We could not find any relation of mitochondrial structure to the phase in the cell cycle as has been reported for the yeast *Candida albicans* (Tanaka and Kanbe, 1985). Our provisional conclusion about the spatial organization of the mitochondria in the organism is that they are dynamical structures able to form both spheres and network like structures, depending on growth conditions.

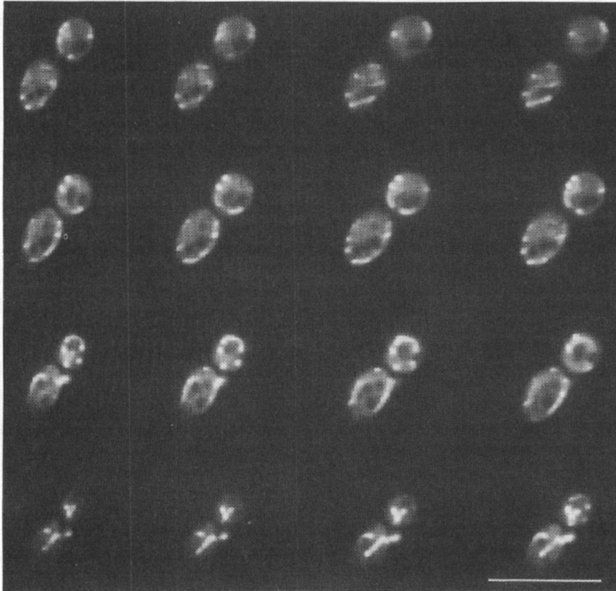


Fig. 2. 16 Sections through a cell of the yeast *Saccharomyces cerevisiae*, taken at 640 nm. intervals. These sections resemble in their appearance the traditional technique of making serial sections in electron microscopy, however, the cell remained alive during this procedure. The sections are arranged from top-right (first section) to bottom-left (last section). Bar = 5 μm.

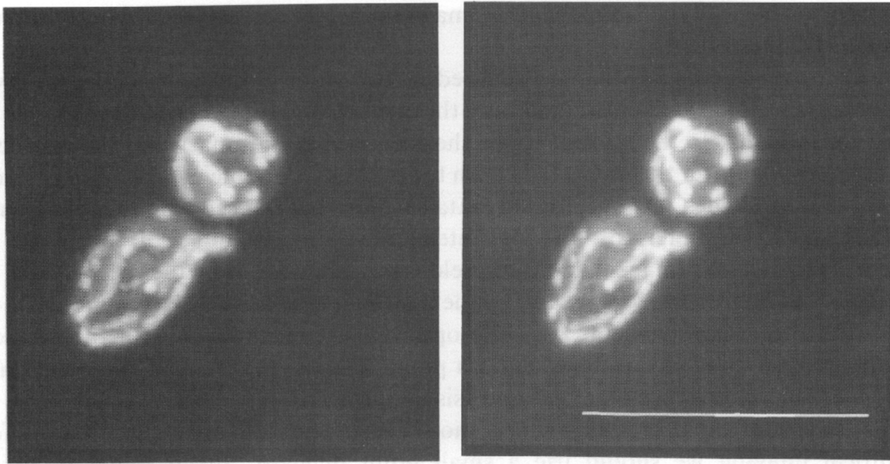


Fig. 3. Stereoscopic image pair, constructed from the 16 slices shown Fig. 2. Note the network-like appearance of the mitochondria, which does not show up in the individual sections. Bar=5 μm.

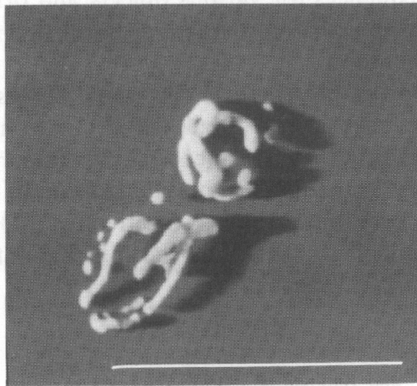


Fig. 4. In the S.F.P. (see text) rendering of the data of Fig. 1 a direct and solid-model like impression of the 3-D organization of the mitochondrial structure is created. Bar=5 μm.

3-D image representation is in the form of a series of sections (Fig. 2), a pair of stereoscopic images (Fig. 3) and in the form of a S.F.P. rendering (Fig. 4).

CONCLUSION AND COMMENTS

A confocal microscope can be viewed as a 3-dimensional microscope. The sectioning aspect plus the high lateral resolution result in the property that in first approximation data are collected only from the volume element in specimen space as defined by the confocal optics. Coupled to a computer system a confocal microscope can now be considered as creating a three dimensional image of the specimen in a three dimensional computer array. In contrast conventional micros-

copy forms basically a 2-dimensional image on a 2-D carrier like a photographic plate or CCD-array.

Confocal microscopy can be realized in two modes. In the so called on-axis mode the specimen is scanned, and only the central, on-axis point of the lensfield is used for imaging. In the off-axis mode the specimen is stationary and the confocal beam is scanned over the image field with for instance a double mirror system. Each of these approaches has specific advantages. For high speed applications beam scanning is to be preferred. On-axis systems have the advantage of being able to address much larger image fields. This field is not determined by the field of view of the optics as in off-axis systems, but by the in principle arbitrarily large amplitude of the specimen scanmovement. Also the optical imaging conditions in the on-axis mode are exactly identical for each data point. This is important if these data are used later in image processing and analysis applications.

The size of the detection pinhole should be determined with care. Ideally for confocal imaging we should use a small point detector. From signal to noise considerations this is often not practical, especially in fluorescence, and a finite size detector has to be used. The influence of the size of the pinhole on imaging have been considered by Carlini *et al.* (1987) and Van der Voort *et al.* (1988). The latter evaluates also the consequences for various objects and orientations. The general effect of enlarging the pinhole is that the lateral resolution is affected at an earlier stage than the axial resolution.

Confocal microscopy as presented in this paper shows that high-quality 3-D images of biological specimens can be obtained in this technique. We expect, also based on our experiment with the instrument over a number of years, that confocal microscopy will be very valuable for the elucidation of 2-D and 3 biological structures. This will be even more the case if effective 3-D image processing and analysis routines are available to operate on the confocal data.

REFERENCES

Brakenhoff, G. J., Blom, P., and Barends, P., 1979: Confocal scanning light microscopy with high aperture immersion lenses. *J. Microsc.* **117**, 219–232.

Brakenhoff, G. J., Blom, P., and Woldringh, C. L., 1980: Developments in high resolution confocal scanning light microscopy (CSLM). In *Scanned Image Microscopy* ed. E. A. Ash. Academic Press.

Brakenhoff, G. J., van der Voort, H. T. M., van Spronsen, E. A., Linnemans, W. A. M., and Nanninga, N., 1985: Three-dimensional distribution in neuroblastoma nuclei shown by confocal scanning laser microscopy. *Nature* **317**, 748–749.

Bereiter-Hahn, J., 1976: Dimethylaminostyrylmethylpyridiniumiodine (DASPMI) as a fluorescent probe for mitochondria *in situ. Biochim. et Biophys. Acta* **423**, 1–14.

Bereiter-Hahn, J., Seipel, K. H., Voth, M., and Ploem, J. S., 1983: Fluorometry of mitochondria in cells vitally stained with DASPMI or rhodamine 6G0. *Cell Biochem. and Function*, **1**, 147–155.

Carlini, A. and Wilson, T., 1987: The role of the pinhole size and position in Confocal Scanning Image System. *Proc. Spie.* **809**, 97–100.

Carlsson, K., Danielson, P. E., Lenz, R., Liljeborg, A., Majlof, L., and Asland, N., 1985: Three-dimensional microscopy using a confocal scanning laser microscope. *Opt. Lett.* **10**, 53–55.

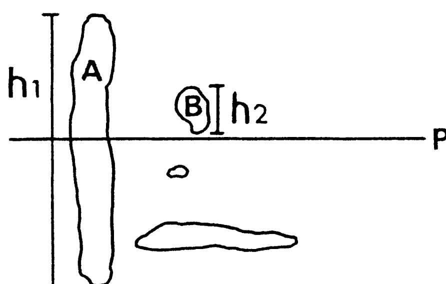
Egger, M. D. and Petran, M., 1967: New reflected light microscopy for viewing unstained brain and ganglion cells. *Science* **157**, 305–307.

Grimes, G. W., Mahler, H. R., and Perlman, P. S., 1974: Nuclear gene dosage effects on mitochondrial mass and DNA. *J. Cell. Biol.* **61**, 565–574.

Hoffmann, H. P. and Avers, C. J.: Mitochondrion of yeast: ultrastructural evidence for one giant, branched organelle per cell. *Science* **181**, 749–751.
Sheppard, C. J. R. and Choudhury, A., 1977: Image formation in the scanning microscope. *Opt. Acta* **24**, 1051–1073.
Stevens, B., 1977: Variation in number and volume of the mitochondria in yeast according to growth conditions. A study based on serial sectioning and computer graphics reconsitution. *Biol. Cellulaire* **28**, 37–56.
Tanake, K. and Kanbe, T., 1985: Three-dimensional behaviour of mitochondria during cell division and germ tube formation in the dimorphic yeast Candida albicans. *J. Cell Sci.* **73**, 207–220.
Van der Voort, H. T. M., Brakenhoff, G. J., Valkenburg, J. A. C., and Nanninga, N., 1985: Design and use of a computer controlled confocal microscope. *Scanning* **7**, 66–78.
Van der Voort, H. T. M., Brakenhoff, G. J., and Baarslag, M. W., 1988: Three-dimensional visualization methods for confocal microscopy. *J. Microscopy* (to be published).
Van der Voort, H. T. M. and Brakenhoff, G. J., 1988: Modelling of 3-D confocal imaging at high numerical aperture in fluorescence. *SPIE*, in press.
Wijnaendts van Resandt, R. W., Marsman, H. J. B., Kaplan, R., Davoust, J., Steltzer, E. H. K., and Sticker, R., 1985: Optical fluorescence microscopy in 3 dimensions: microtomoscopy. *J. Microsc.* **138**, 29–34.
Wilson, T. and Sheppard, C. J. R., 1984: *Theory and Practice of Scanning Optical Microscopy.* Academic Press, New York.
Woldringh, G. L., DE Jong, M., Van den Berg, W., and Koppes, L., 1976: Morphological analysis of the division cycle of *Escherichia coli* substrains during slow growth. *J. Bacteriol.* **131**, 270–279.

DISCUSSION

Q. When you select a cell from a 3-D space, how do you do it? If the optical section is simply moved to some position in the space then it will hit 'big' things with higher probability than 'small' ones. (In this case, 'big' is defined as height normal to the section.)



It is more probable that A will be hit than B by the plane P by the ratio h1/h2. To select particles for measurement with uniform probability you must use a uniform selection principle e.g. the dissector principle. Which did you use?

(Howard, C. V.)

A. It is indeed important to avoid a bias in the selection of the objects. Probably principles derived from stereology should be applied, but we have not done that up to now.

Q1. What is a typical scan time?

Q2. Can you use it on wet, living specimen? And do you have problems with the Brownian motion?

Q3. Can you please make a comment on the preparation method of the wet cells? This may be interesting to use, as we are preparing wet cells for x-ray microscopy, where we have exposure times of a few seconds and need to use very thin water layers. (Niemann, B.)

A1. With scan time frequencies of 50–200 Hz the 2-D image collection time is typically 1 to 5 seconds. A 16 section 3-D image takes about 80 seconds.

A2. Yes, confocal microscopy is especially suited for use on hydrated objects. Yes, if objects are not embedded in a medium of sufficient viscosity.

A3. We examine basically a layer of 100 to 200 μm of an object which may be much thicker. We therefore do not have the problem indicated.

Q1. I am much impressed by your work.
Is your technique influenced much by the refraction of light?

Q2. If the distribution of the modulus of refraction in the material is not known, how do you calibrate? (Takaki, R.)

A1. Refraction of light in the specimen may affect the imaging. The degree will depend on the differences in refractive index in the material. For biological objects of reasonably uniform composition, refraction effects do in general not affect the imaging.

A2. We have not addressed this problem.