PERGAMON

Micron 32 (2001) 449–453

micron

www.elsevier.com/locate/micron

Short communication

Computer-generated, three-dimensional reconstruction of histological parallel serial sections displaying microvascular and glandular structures in human endometrium

F. Manconi[a,*], R. Markham[a], G. Cox[b,c], E. Kable[b,c], I.S. Fraser[a]

[a]*Department of Obstetrics and Gynaecology, Queen Elizabeth II Research Institute for Mothers and Infants, The University of Sydney, Sydney, NSW 2006, Australia*

[b]*The Australian Key Centre for Microscopy and Microanalysis, The University of Sydney, Sydney, NSW 2006, Australia*

[c]*The Electron Microscope Unit, The University of Sydney, Sydney, NSW 2006, Australia*

Received 12 March 1999; received in revised form 15 February 2000; accepted 22 February 2000

Abstract

This paper describes a technique to develop high-resolution three-dimensional (3D) images of microvasculature structures in curettage, hysterectomy or endometrial resection biopsies using parallel histological serial sections. Employing a labelled streptavidin–biotin–alkaline phosphatase (LSAB$^+$) method and visualising by using DAB$^+$ with the primary antibody, mouse anti human Q-Bend-10, the images were directly digitised from a light microscope into the KS400 Universal Image Processing and Analysis software via a CCD colour camera; binary images of the structures were created and the binary images were exported into VoxBlast 3D rendering software to view still and rotating 3D images on a computer monitor. This in turn enabled hard copies of the full sequence to be printed.

Keywords: 3D computer reconstruction; Microvascular; Endometrium; Kontron; VoxBlast

1. Introduction

The history of 3-dimensional reconstruction (3DR) has been well documented in Gaunt and Gaunt (1978). Computer-aided 3DR was first introduced by Glaser and Van Der Loos (1965) to trace the structures of Golgi-filled neurons. A review of fifty-eight different software packages, using an assessment system that included 173 items for computer aided 3DR, was prepared by Huijsmans et al. (1986). Many laboratories have been participating in developing software for the study of 3D structural biology (Hessler et al., 1992, 1996; Kam et al., 1992; Leith, 1992).

Approaches to a semi-automated 3DR have continued to rely on photography for data storage and use lower resolution digitisation for computer-assisted reconstruction. It is now possible to use direct, computer-based image capture and processing to eliminate the use of film and to decrease the labour involved in 3DR of objects from light microscopy serial sections.

3DR studies in the area of gynaecology are few; there are a number of recent papers dedicated to computer assisted 3DR of the endometrium (Donnez et al., 1992, 1994; Casanas-Roux et al., 1996; Huang et al., 1996).

With the improvements in both computer hardware and software engineering tools, computerised modelling of anatomical and histological morphologies has become very useful for visualising complex 3D forms. Computer models not only provide a means to visualise complex morphology derived from two-dimensional (2D) tissue outlines, they also permit mathematical modelling of growth or function attributes not otherwise observable.

There are many surface rendering techniques to display structural data obtained by image capturing devices in the area of 3D; when comparing this to volume rendering there are fewer techniques available for obtaining the same outcome. Udupa et al. (1991) concluded from a comparison that in the current state of development the surface rendering methods had a slight edge over the volume rendering methods. Further recent studies by White (1995) and Cox (1999) discuss visualisation and quantification using both voxel and surface rendering approaches.

Today, using off-the-shelf products, researchers can investigate microvasculature structures in 3D relatively inexpensively. All that is required is access to a personal

* Corresponding author.

0968-4328/01/$ - see front matter
PII: S0968-4328(00)00012-3

computer, SVGA monitor, CCD camera, a collection of 2D images, image analysis processing system and volume visualisation software. Since most of the hardware consists of general purpose computer equipment, its cost can easily be justified by other laboratory uses.

2. Materials and methods

Endometrial samples used for the study were selected from patients of reproductive age who had curettage, hysterectomy or endometrial resection in the King George V and the Royal Prince Alfred Hospitals, Sydney, Australia. The Ethics Review Committee of the Central Sydney Area Health Service approved collection of the samples for research purposes. Samples were only included if subjects had no specific gynaecological disease and had not undergone recent hormonal treatment. Endometrial samples were blindly assessed by a specialist gynaecological pathologist and accurately dated by the criteria of Noyes et al. (1950). Blocks from 12 subjects were examined (3 early secretory, 3 late secretory, 3 proliferative and 3 menstrual phases).

2.1. Immunohistochemistry

Routinely processed specimens (formalin–acetic acid–alcohol (FAA) fixed, paraffin embedded) were serially sectioned and 20–30 sections (5 μm thickness) were cut on silanised slides. The slides were deparaffinised in histolene and rehydrated through successive concentrations of ethanol, to tap water and Tris-Buffer Saline (TBS) for 5 min. Non-specific (background) staining was reduced by pre-incubating (5 min) with normal swine serum (1:5 dilution). A humidity chamber was used for the primary antibody incubation (mouse anti Q-Bend-10) (Novacastra, Newcastle upon Tyne, UK), 1:500 dilution, 30 min at room temperature. The immuno-stain was performed using the labelled streptavidin–biotin–peroxidase (LSAB$^{®+}$) kit (K0690, Dako Corporation, Carpinteria, USA) and visualised using the 3,3′-diaminobenidine substrate chromogen system (DAB^{+}) (K3468, Dako Corporation, Carpinteria, USA). The slides were then counter-stained in Mayer's Haematoxylin, coverslipped with Ultramount (Histo-Labs, Riverstone, Australia) and the slides were examined by light microscopy (Olympus BH-2, Olympus, Tokyo, Japan). Appropriate positive and negative controls were also used as an aid in the assessment of antibody specificity. All brown-black coloured structures were considered as positive even if a lumen could not be identified (Fig. 1).

2.2. Image analysis

The immunohistochemical stained sections were viewed with a Zeiss Axioplan optical microscope (Carl Zeiss GmbH, Jena, Germany), using a 40× NA 0.75 objective lens. The images were directly captured from the microscope into the KS400 Universal Image Processing and Analysis software (Carl Zeiss Vision GmbH, Munich, Germany). Once the primary image had been selected, the gland tissue was segmented using the 'segment region' option within KS400, and a binary template was placed in the overlay plane. The following slide could then be oriented precisely to match, using the rotation stage of the microscope. As we discuss later, this provides a more precise registration than using fiduciary marks since it is unaffected by the inevitable distortions of sectioning. Once all the images had been collected, separate binary images of the glands and microvessels were extracted (Figs. 2a and b). All images were initially acquired at a resolution of 768×576 pixels to enable accurate registration and segmentation but once this was done the images were reduced to half the size (384×288 pixels). This provided the dual benefits of making the data set more manageable for 3DR and reducing the discrepancy between the voxel size in the *XY* dimension and in depth. The latter parameter was an inevitable consequence of the methodology, since 5 μm is the thinnest practicable section thickness for microtomy of paraffin-embedded material. Even so, a 15-fold expansion in the vertical dimension was required to make the voxels isotropic. This was achieved by replicating individual sections 15 times. Replication is the only way to ensure volumetric accuracy. While for visual quality other techniques might have advantages, in practice linear interpolation is the only commercially available alternative. This algorithm fails completely in samples such as these, since the data is binary in nature and interpolation is therefore impossible.

2.3. Three-dimensional reconstruction

The binary images constructed in the KS 400 were then rendered as individual data sets in VoxBlast (Vaytek Inc, Fairfield, IA, USA). VoxBlast is a volume rendering program, designed as a general purpose rendering application for research. VoxBlast accepts stacks of registered 2D images and creates 3D projections from any viewpoint using an alpha blending or surface rendering algorithm. Tools included are for lighting, 2D and 3D measurements, 2D slice viewing and movie loop generation. Since we were rendering solid surfaces the post rendering lighting model was chosen after the images had been rendered. The reconstructed 3D image models of the endometrial vessels were displayed with the microvessels in red and the glands in green. Vessels overlying the glands appeared as bright yellow and those behind the glands as faint yellow (Fig. 3). The 3D images could then be displayed as solid or transparent structures, the reconstruction could be rotated and viewed in any direction. A sequence of views could be created by using the movie loop generation function to render a specified number of frames, which could then be played as a continuous movie.

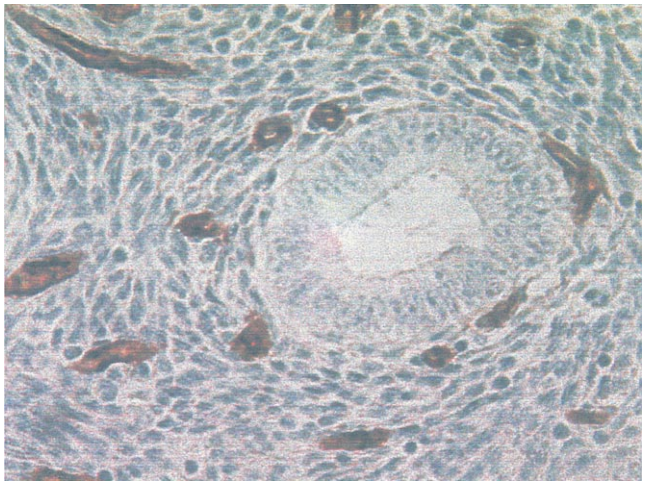

Fig. 1. Immunohistochemically stained endometrial section, CD34 (400×).

3. Results

The results can be seen in Figs. 1, 2 and 3.

4. Discussion

This paper describes the development of a method for computed 3DR of the human endometrial microvasculature. Viewing of a rotating image on a computer monitor or recorded and later played back on a video recorder gives a clear understanding of 3D relationships of glands and microvessels; this technique provides excellent visual cues to the shapes of different structures. Single computer print outs or photographs of a 3D image rarely provide an impression of the three-dimensionality of the structure. Three dimensional reconstruction of microscopic structures is useful for demonstrating the shape and size of structures, for showing relationships between groups of cells, for demonstrating connection between structures and for performing volumetric measurements on tissues that

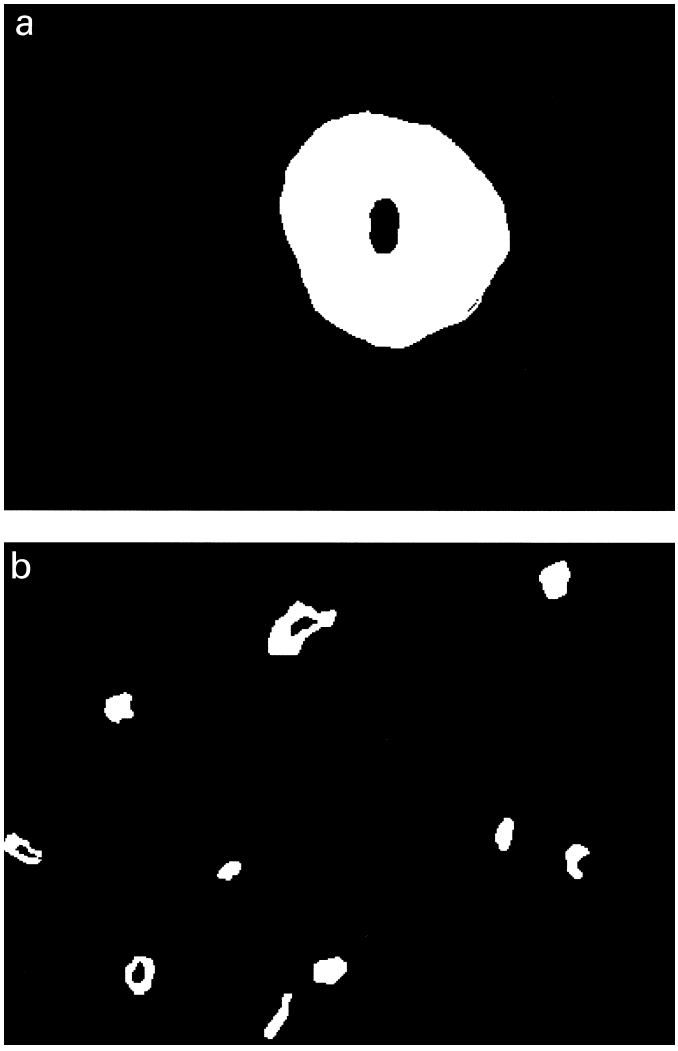

Fig. 2. (a) Binary image of gland. (b) Binary image of microvessel.

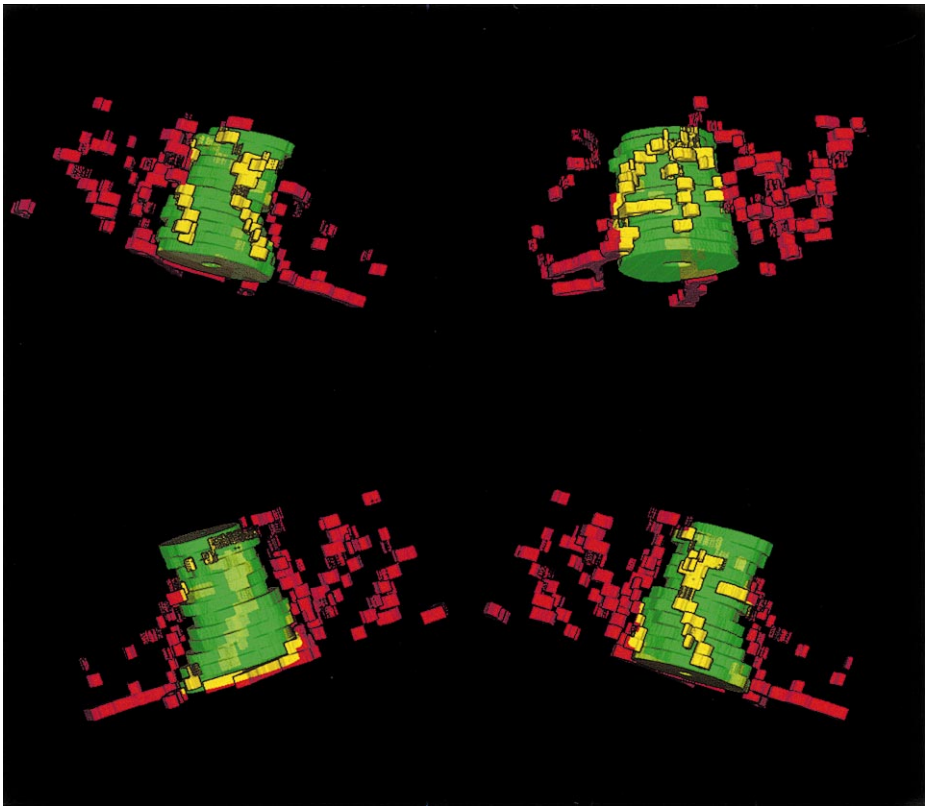

Fig. 3. A 4-way image of the 3D reconstruction of a gland and surrounding microvessels. The gland is shown in green and microvessels in red and microvessels overlaying the glands appear as bright yellow and microvessels behind the gland as faint yellow.

would not normally be suitable for examination because of their small size. There are several reasons for choosing computer reconstruction: volumetric calculation, measurement of structures and assessing precise relationships of structures.

The introduction of artificial fiducial markers is appropriate and useful in light microscopy. Our method for registration did not rely on fiducial markers rather the intrinsic structures of several objects (glands, epithelium or solitary vessels). The use of non-fiducial, shape-based registration of biological tissue is described by Montgomery and Ross (1996). A study conducted by Jones et al. (1994) noted that dimensional changes in tissue occurred due to micrometry in all the sections that were measured. We recognise the necessity to determine tissue distortion, but careful best-fit manual registration of several objects on each successive section on a sequential basis partially compensates for small degrees of distortion.

Fixation and dehydration can result in tissue shrinkage, while sectioning can introduce compression artifact. Stretching can also occur during "floating out" time and temperature of the water bath, these can cause difficulties in image 'alignment' or 'registration' between successive histological sections so the above steps should be kept constant. It was also found that registration was made easier if all the sections were orientated similarly on the slides and a rotating stage was used at the time of data acquisition. Deverell et al. (1989) acknowledged the fact that tissue distortion, which resulted from processing and embedding, would significantly affect the size and shape of 3DR, making it unrealistic to perform volumetric studies on microscopic structures unless all aspects of tissue preparation are standardised and documented.

Fully automatic segmentation was not achievable on the tissue samples that we were able to obtain; an alternative method was the seed fill method. Our data sets were sparsely sampled in the Z dimension and consisted of binary images. In these circumstances linear interpolation fails completely since there is either nothing or the same grey-value to which to interpolate. Slice replication, on the other hand, is totally objective and is the most intrinsically accurate means of linking successive sections. In fact even in less sparse grey-scale, data replication is more objectively accurate—linear interpolation is used mainly to obtain a less blocky looking image.

The manual work involved in segmenting out accurate binary images of the glands and microvascular vessels remains the most time-consuming aspect of this work. However, the complexity of the samples is such that the 3DR of 24-bit colour images are not readily comprehensible. Labelling techniques which are more easily discriminated by both eye and machine, such as fluorescent probes, may offer a future solution to this problem.

The chromogen DAB^{+} was used for visualisation as it is

stable and therefore ideal for archival purposes, and mouse anti Q-Bend-10 was chosen as the primary antibody owing to the fact that it is a surface glycophosphoprotein, which is expressed by virtually all small-vessel endothelial cells (Krause et al., 1996). This computerised technique with immunohistochemical localisation of microvascular structures has allowed the precise assessment of size, shape, interrelationship and interconnections between microvessels at different depths in the endometrium and their relationships to glands and to surface epithelium.

In conclusion, the KS400 Universal Image Processing and Analysis software together with VoxBlast give an example of a computer visualisation system that is capable of excellent 3DR from a serially sectioned material for scientific interpretation and measurement or demonstration purposes. It is clear that computer assisted 3DR will continue to remain a research tool for sometime, when used for visualisation purposes it could easily be applied to study angiogenesis in tumours, the mode of action of new vascular inhibitors in experimental treatment of tumours. Also in organs where the micro-anatomy vitally influences function and dysfunction, e.g. central nervous system, lung and liver are also likely to be fertile grounds for this type of investigative approach.

References

Casanas-Roux, R., Nisolle, M., Marbaix, E., Smets, M., Bassil, S., Donnez, J., 1996. Morphometric, immunohistological and three-dimensional evaluation of the endometrium of menopausal women treated by oestrogen and crinone, a new slow-release vaginal progesterone. Human Reproduction 11, 357–363.

Cox, G., 1999. Equipment for mass storage and processing data. In: Conn, M.P. (Ed.). Methods in Enzymology, Academic Press, San Diego, CA, pp. 29–55.

Deverell, M.H., Bailey, N., Whimster, W.F., 1989. Tissue distortion in three-dimensional reconstruction of wax or plastic embedded microscopic structures. Pathology and Research Practice 185, 598–601.

Donnez, J., Nisolle, M., Casanas-Roux, F., 1992. Three-dimensional architectures of peritoneal endometriosis. Fertility and Sterility 57, 980–983.

Donnez, J., Nisolle, M., Casanas-Roux, F., 1994. Peritoneal endometriosis: two-dimensional and three-dimensional evaluation of typical and subtle lesions. Annals of the New York Academy of Sciences 734, 342–351.

Gaunt, W.A., Gaunt, P.N., 1978. Three Dimensional Reconstruction in Biology, Pitman Medical, Tunbridge Wells, Kent, England.

Glaser, E.M., Van Der Loos, H., 1965. A semi-automatic computer microscope for the analysis of neuronal morphology. IEEE Transactions on Biomedical Engineering 2, 22–31.

Hessler, D., Young, S.J., Ellisman, M.H., 1996. A flexible environment for the visualization of three-dimensional biological structures. Journal of Structural Biology 116, 113–119.

Hessler, D., Young, S.J., Carragher, B.O., Martone, M.E., Lamont, S., Whittaker, M., Milligan, R.M., Masliah, E., Hinshaw, J.E., Ellisman, M.H., 1992. Programs for visualization in three-dimensional microscopy. Neuroimage 1, 55–68.

Huang, L., Chen, L., Chen, Q., 1996. Three dimensional reconstruction of human endometrial spiral arteries preinsertion and postinsertion of IUD. Chung-Hua Fu Chan Ko Tsa Chih (Chinese Journal of Obstetrics and Gynecology) 31, 523–525.

Huijsmans, D.P., Lamers, W.H., Los, J.A., Strackee, J., 1986. Toward computerized morphometric facilities: a review of 58 software packages for computer-aided three-dimensional reconstruction, quantification, and picture generation from parallel serial sections. The Anatomical Record 216, 449–470.

Jones, A.S., Milthorpe, B.K., Howlett, R., 1994. Measurement of microtomy induced section distortion and its correction for 3-dimensional histological reconstructions. Cytometry 15, 95–105.

Kam, Z., Chen, C., Sedat, J., Agard, D., 1992. Analysis of three-dimensional image data: display and feature tracking. In: Frank, J. (Ed.). Electron Tomography, Plenum, New York, pp. 237–256.

Krause, D.S., Fackler, M.J., Civin, C.I., Stratford, W.M., 1996. CD34: structure, biology and clinical utility. Blood 87, 1–13.

Leith, A., 1992. Computer visualization of volume data in electron tomography. In: Frank, J. (Ed.). Electron Tomography, Plenum, New York, pp. 215–236.

Montgomery, K., Ross, M.D., 1996. Non-fiducial, shape-based registration of biological tissue. SPIE Electronic Imaging 2655, 224–232.

Noyes, R.W., Hertig, A.T., Rock, J., 1950. Dating the endometrial biospy. Fertility and Sterility, 3–25.

Udupa, J.K., Hung, H.M., Chuang, K.S., 1991. Surface and volume rendering in three-dimensional imaging: a comparison. Journal of Digital Imaging 4 (3), 159–168.

White, N.S., 1995. Visualization systems for multidimensional CLSM images. In: Pawley, J.B. (Ed.). Handbook of Biological Confocal Microscopy, Plenum, New York, pp. 211–254.