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Invited Review

Three-dimensional reconstruction in microscopical morphology

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Summary. This article reviews the current status of three-dimensional reconstruction as a tool in the understanding of microscopical morphology. Many microscopical structures have a three-dimensional shape that cannot readily be appreciated by the study of sections alone. Three-dimensional reconstructions often provide novel information about such structures and enable researchers to formulate new hypotheses about the subject of their study. This review concentrates primarily on the methodology involved and how it is used to generate three-dimensional images and to provide three-dimensional quantitative data.

Key words: Three-dimensional reconstruction, Microscopy, Morphology

Introduction

The loss of the third dimension is an inherent problem with examining sectioned material when the tissue to be examined possesses a complex morphology (Weibel and Elias, 1967). When three dimensional (3-D) information about numbers, directions or probabilities is required, stereology can compensate for the loss of one dimension by reconstruction of the average spatial relationships on theoretical grounds (Weibel and Elias, 1967). However, when the structure itself must be examined three-dimensional reconstruction (3RD) is required. This allows the third dimension to be studied directly by the displaying of 3-D models or static or kinematic images. In recent years, the powerful combination of qualitative 3-D structural reconstruction and 3-D quantitation has permitted the study of biological processes on a measurable basis and, because of this, 3DR is rapidly permeating many areas of basic and applied biology. This review commences with a description of the aims of 3DR, but focusses primarily on the methodology of 3DR, for both tissue (serial) and confocal (optical) sections, and on image presentation and 3-D quantitation. Some examples of applications of 3DR in histology and histopathology are then briefly addressed, before the review concludes with some thoughts on the future of 3DR.

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Objectives

In terms of the biological question that is being addressed, the purpose of each 3DR will normally be unique. Nevertheless, it is usually possible to think of most 3DR as falling into one of the following descriptive categories.

1. The visualisation and understanding of the anatomical boundaries of structures in three dimensions, particularly those that are «hidden», e.g. an individual nerve fibre from within a bundle.

2. The examination of the relationships between components, such as whether they are connected or not e.g. neurones in the cortex (Rydmark et al., 1992) or notochordal tissue in embryos (Salisbury et al., 1993).

3. The analysis of the distribution of components: this can be at a tissue level, e.g. lymphoma deposits in bone marrow trephines; at a cellular level, e.g. ALIPs (Abnormal Localization of Immature Precursors) in the bone marrow of patients with myelodysplastic syndromes; or at a subcellular level, e.g. the 3-D positions of chromosomes within a cell nucleus.

4. The localization of entities, e.g. dopamine-containing neurones in the human mid-brain (Woodward, 1983). Co-localization studies of antigens demonstrated by immunocytochemical techniques also fall into this category e.g. labelling two cytoskeletal elements with antibodies visualized by different chromagens.

5. Spatial quantitation, such as object counting; cell density histograms e.g. the 3-D distribution of cell numbers in different regions of the brain (Woodward, 1983); and the determination of volumes occupied by reconstructed structures.

6. Intensity measurements. 3-D is considerably better

than 2-D for these assessments because there is no section thickness artefact. Although the use of 3-D improves the quantification of intensity, it also introduces a new set of problems. Bogler et al. (1993) give a list of the checks and controls required when attempting the quantitation of fluorescent emission from immunofluorescently labelled specimens.

Methodology

1. 3DR from serial tissue sections

Requirements

The requisites for 3DR have been discussed by Whimster and Cookson (1991) and can usefully be considered in stages: (1) the acquisition of the structure to be examined, (2) the preparation of thin 2D sections, collected so that true alignment or registration of the stack of images can be achieved, (3) the isolation and orientation of the structure to be reconstructed (4) data entry and (5) image display. These stages can provide a framework for examining both how 3DR is done and how recent advances have had an impact in this field.

(1) Acquisition of the structures to be reconstructed

For most biological structures, acquisition of the structure to be reconstructed poses relatively few problems. Whole organ slices may reveal the structures of interest and allow reconstruction fairly easily e.g. reconstruction of cirrhotic liver nodules (Yaegashi et al., 1987). For microscopic slices (serial tissue sections) there is, clearly, a size limitation.

(2) Preparation of the 2D slices with true alignment or registration of the stack of images

Preparation of the stack of 2D images has to be done with some care. Fleege et al. (1991) have investigated the influence of variations in tissue processing, especially of fixatives, on morphometry. Fixation procedures generally cause tissues to shrink e.g. standard formalin fixation may shrink tissues by up to 30%; this is well known to pathologists who may examine frozen and paraffin sections from the same case. A constant factor may have to be introduced into the reconstruction to allow for this. Serial tissue sections can be readily cut from a paraffin block, containing the structure of interest, but variable stretching of the tissue sections occurs whilst they are being floated on the water bath (Deverell et al., 1989a). This means that the temperature of the water bath has to be kept constant and the same flotation time allowed for each section. Similar considerations apply to resin sections. The amount and type of staining will influence the size of structures detected (Perkins et al., 1979; Deverell et al., 1989b), as will variation in section thickness due to the microtome slicing. Clearly, if the reconstruction is to be used to acquire 3-D quantitation data, these variations may have to be properly corrected or their distorting effect reduced to sampling resolution size. Mounting procedures can also introduce deformations into the serial sections and have to be carefully standardised.

The serial sections have to be accurately orientated; this process is known as image registration. It is necessary to prevent progressive errant shift of the stack of serial sections caused by translational misalignment between sections in the x, y plane and/or rotational misalignment about the z-axis. Image registration can be achieved in two ways, either by the introduction of registration markers (fiducials) or by the use of computer overlays. Registration markers of various types have been used. These have included structures that can be embedded along the primary tissue and sectioned with it, such as lengths of nerve, and holes drilled perpendicular to the slicing plane into the wax or resin block, either manually (Gough, 1967) or with a laser (Hamilton, 1989, quoted by Cookson, 1994), sometimes with subsequently inserted nerve fibres (Street and Mize, 1983). Useful registration markers for tissues embedded in paraffin blocks are cactus spines (Mammillaria species) passed into the tissue before embedding in wax (Deverell and Whimster, 1989). These section cleanly and can be readily located in the tissue sections by their birefringence when examined under polarized light. For hard tissues, registration can be achieved by three V-shaped grooves, two parallel and one diagonal, cut or etched into the rectangular side(s) of the block (Kimura et al., 1977).

Registration markers can be replaced by computer overlays in certain situations. These computer overlays can either be interactive (image analysis-based) or automatic (computer-based). Comparison of the two methods for image registration of consecutive light photomicrographs of nerve tissue (Rydmark et al., 1992) has shown that interactive procedures (based on a subjective «best fit» are faster but less robust (because of the operator's subjective expectancy). Automatic registration by image thresholding and binary comparison is slower (4-16 images per hour) but results in a truer, non-subjective, positioning. For structures where the external shape is known, composite photomicrographs (transparencies) of the tissue with a registration micrometer scale can replace drawings, registration marks and calibration scales. These can be made using a microscope which allows for the simultaneous attachment of a video camera and a standard photographic camera. A data set describing the first section on the microscope stage is captured, via the video camera and visualized using image analysis equipment, when interactive contouring can be carried out. A registration micrometer scale is then placed on top of the section and its image also contoured around. Both contours are then stored as a computer overlay and a composite photomicrograph taken with a standard camera. The second section is placed on the microscope stage and aligned with the computer overlay of the

contour of the first section. The registration micrometer scale is placed on top of the aligned second section and aligned with its previous computer overlay. A composite photomicrograph is then taken of the aligned second section with the micrometer scale, and the process is repeated until the end of the section stack. The transparencies can then be projected using a standard slide projector, attached to a macro stand, onto a digitiser tablet either to make registered drawings or for direct data entry (Deverell et al., 1993).

(3) Isolation and orientation of the structure to be reconstructed

Selection from the images of the components to be reconstructed usually involves enlargement of the components. It can be done manually by making drawings and this has some advantages: (i) The task of making the drawings itself often serves to focus concentration on the structure. Even before completion of the 3-D model, this may bring a better understanding of the anatomical boundaries of the components of the structure and their connections with each other. For some 3DR programs, this knowledge of what connects to what is a pre-requisite at the data input stage. (ii) Drawings may permit a number of internal structures to be reconstructed at different times with reference to another structure e.g. the external shape of an embryo. (iii) Drawings are easily carried out and require no specialised equipment. Drawings are, however, very labour intensive and, in many countries, labour is expensive.

The alternatives to drawings are either interactive selection of the structure of interest on the computer monitor (Woodward, 1983) or automatic segmentation by the computer using image processing methods (Russ, 1990), for example, thresholding to reveal the structure of interest or by tracing the boundary of the object starting from an electronic «seed».

(4) Data entry

Entry of the selected image data (the co-ordinates for x, y, and z, i.e., length, width, and thickness or depth) into the computer can be done by contouring around the structure of interest. The computer automatically samples the contour to derive a number of coordinate data points which define its boundary. The coordinate points can also be input by depressing a button on the cursor on the digitiser tablet at a number of selected points on the outline. This sounds more awkward than contouring but in practice is considerably easier. It does however lead to the next consideration which is the amount of data to input.

The sampling distance should be based on the objects being examined and be at least two and a half times smaller than the size of the smallest detail of interest (Shannon, 1949). False detail can appear in the images if the sampling distance is larger than this. With large objects, this leads to the incorrect interpolating of their outline, and with small objects results in an over-estimation of their number.

Optimising the number of slices per reconstruction ensures uniform sampling of points in three dimensions whilst allowing for the rate of change of profile of the structure under reconstruction. A simple equation for the optimal number of z-plane slices ($n_{z\text{-plane}}$) is given by:

$n_{z\text{-plane}} = n_z + \Sigma(\delta y)$

where $n_z = n_{dpl} \cdot d/2L$

and $\delta y = (y_2 - y_1)/ST$

(n_{dpl} is the number of data points for the largest section in the reconstruction, d is the depth of the structure (μm), L is the maximum dimension (μm) of the largest section, y_1 is the maximum dimension of any one section, y_2 is the maximum dimension of its subsequent section and ST is the section thickness given by d/n_z) (Deverell et al., 1993). An alternative equation, with the loss of several terms, has been proposed (A. Entwistle, personal communication): the optimal number of sections = the number of data points in the largest section (n_{dpl}) multiplied by one plus the sum of the differences between the maximum dimensions of subsequent sections, or,

$n_{z\text{-plane}} = (n_{dpl}) \cdot (1 + \Sigma(\Delta y_1)/2.L)$.

These equations are applicable in situations where the external shape of the object under reconstruction is known because the 3-D computer-generated images can be compared directly with measurements and photographs taken prior to sectioning. Other methods of interpolating between z-plane slices may be preferable when the external shape is not known (Pentland, 1984; Barnsley, 1988; Cross et al., 1994).

The handling of the raw data and the modelling of the reconstructed image by the computer are in the province of mathematicians, statisticians and computer scientists and fall outside the scope of this review. Much of the progress that has been made 3DR is however due to their efforts and anyone seriously interested in 3DR is advised to collaborate with them whenever the opportunity arises. Huijsmans et al. (1986) have discussed computer-aided 3DR and derived a set of recommendations that can be helpful when selecting packages.

It is important to realise that the size of image files can be a limiting factor. Image resolution is expressed in terms of pixels or picture elements. To store 256 levels of grey values (a grey scale) for one pixel requires one byte. To store a 2D array of pixels i.e. a full image, for example, 512 x 512 pixels requires 0.25 megabytes of storage. For microscopical applications the storage and manipulation of large volumes of data are required. Often this work is performed on high quality work-stations such as SUN or SGI systems. Few packages have been successfully mounted on personal computers. These are often very limited in the amount of data they can handle. Data stored as contours can be held much more compactly since only comparatively few numbers are needed to define a contour. Sophisticated display techniques for contour data are avilable (Holman et al.,

1989).

(5) Image display

(i) Line drawings. Tree or stick-figure representation of connections between neurones, for example, may be used. The complexity of such displays is obviously low.

(ii) Models. Before the use of computers, 3-D models were made from a number of materials that could be obtained as slices and then stacked e.g. wax slices for models of the inner ear and surrounding temporal bone, expanded polystyrene sheets for a model of a human bronchial gland (Whimster, 1982). This research was very labour intensive and a single model could take 3 months (Whimster, 1982).

(iii) Computer-generated images. Computer-generated images have largely replaced line drawings and models. Using coordinate transformations, they can be rotated, scaled and viewed from different perspectives and the different components of a structure may be individually defined and colour coded. The structures can be displayed as points, as wire frames or nets, or with solid, or semi-transparent surfaces. Hidden line and surface removal are essential in recreating how we perceive structures in real life. Various types of surface shading can be applied to give depth cues; two common types applied to triangles are Gouraud (1971) and Phong (1975) shading, the latter has become the gold standard.

Where collections of slices serially acquired have been stored, knowledge of the thickness of the slices allows the collection to be considered a 3D volume. Each element then has x, y co-ordinate and a z co-ordinate for thickness. These elements are then termed volume elements or voxels. For medical imaging applications a huge number of display and shading methods have been derived, most of which are less effective than Phong shading for surfaces. These range from finding the maximum intensity of the voxels in a 2D array of rays projected through the volume termed the maximum intensity projection. Other methods are based on classification of the contents of each small volume element or voxel. Methods have been applied to the extraction of display of surfaces in such data. Two problems arise from these methods. Firstly, different shading methods provide different 3-D cues, for example, fuzzy gradient shading gives a surface appearance based on properties of small groups of voxels. The cue here is a shape cue. No sense of depth or relative ordering of objects is provided. The maximum intensity projection gives geometric cues of 3-D structures in space but no depth or surface information. Depth shaded images simply classify voxels in terms of solely «depth» or distance. It is very important to realise the nature of the different cues provided or errors in interpretation can follow. Secondly, volume data is often linearly interpolated to give cubic voxels to produce acceptable shadings. In these cases it is important to realise that only a small proportion of the data may represent measured values. Failure to appreciate this can again lead to errors in interpretation.

The two main methods of generating models on a computer video screen are (1) slab-stacking images (e.g. 3-D REC, Kontron Electonik) and (2) surface representations such as triangular tiles e.g. the Cookson-Holman-Dykes program (Holman et al., 1989), polygons or B-spline patches. Slab-stacking is a computerized way of doing what was done in the pre-computer era and the resulting images do look obviously «slab-like». Triangulation methods produce better images because suitable intensity-shading of the triangles that make up the boundary of the model gives the impression of a smooth surface.

Some 3DR programs have been specifically designed for use on personal computers e.g. the Cookson-Holman-Dykes program (Holman et al., 1989). The image generation part of this program, Imgen, is a 3-D rendering program that creates shaded images from geometric models of object surfaces (Holman, 1991). ANALYZE (Robb and Hanson, 1990) is the least expensive of the good 3DR packages that are commercially available. It requires the use of a high performance workstation and is expensive (typically costing twice as much as the workstation). A useful feature of many programs is an «electronic knife» that generates new cross-sections and allows exposure of structures within objects at various cut planes.

2. 3DR from confocal microscopy

Confocal microscopes provide a means of obtaining perfectly registered serial optical sections (Minsky, 1957, 1988; Pawley, 1990). Confocal microscopes which examined the light reflected from specimens were the first to be made, the first to be sold commercially and are still the only confocal microscopes of interest in, for example, the semi-conductor industry. The depth that can be examined within a sample is almost completely dependent on the nature of the sample and the size of the object of interest. When a high degree of spatial resolution is required, however, the depth examined within a sample is limited to 50-100 μm. Cogswell and Sheppard (1992) have described a means of implementing differential interference contrast (DIC) on the confocal microscope. The nature of the DIC images generated, which is quite different to those obtained with transmission DIC, have a format that is perfect for combination with images describing the distribution of fluorescence in a sample. For reasons little understood, these techniques are almost never used but they are excellent methods for examining morphology, even in unstained preparations.

Confocal laser-scanning microscope systems were large and very expensive but the technology opened up an exciting new field by dramatically revealing novel information from biological samples. Semi confocal microscopes such as the tandem scanning instruments or

line scanning machines are smaller, easier to use and permit direct viewing of the specimen through the eyepieces. Sophisticated software with advanced analytical capabilities has been developed by many companies for the acquisition, processing, analysis and presentation of confocal images e.g. ANALYZE (Mayo Clinic), ImageSpace (Molecular Dynamics) and VoxelView.

3-D Quantitation

1. Object counting

Some 3DR packages have object counting as a feature. It is difficult to see that this could ever really be a sensible use of 3DR. Stereology packages (e.g. Digital Stereology from Kinetic Imaging Ltd.) provide the tools for much easier, faster and more accurate estimations of this kind. Direct three-dimensional counting does, however, provide an alternative accurate, simple and reliable way to count cells, nuclei, nucleoli, or other objects in sections of tissue between 8 and 100 µm thick. Cells can be counted and measured in an optically defined volume of tissue, called a «counting box», whose top and bottom sides are located inside the section (Williams and Rakic, 1988).

2. Volume measurements

Many 3DR packages include a program that gives the volume of structures. Slab-stacking programs e.g. can estimate the volume of simple spheroids by calculating the surface area of a single slice and multiplying by the thickness (z-value). An adjustment has to be made for the outer ring if two consecutive slices are of different diameter. The spheroid volume is the sum of the slice volumes. Volumes in a triangulation program such as the Cookson-Holman-Dykes program (Holman et al., 1989) are calculated by projecting triangles onto the x, y or y, z plane. Summation of the volumes of the parallelipipeds so created then gives the volume of the structure under consideration. This method is far more flexible in the kind of shapes it can deal with.

Problems of tissue distortion remain a significant handicap to volume measurements, particularly during fixation and section preparation. Section cutting inevitably leads to some loss of tissue. The x, y and z dimensions of the object (calculated from the size, number and thickness of the sections) should correspond to the dimensions of the 3-D model. Constant factors can be used to make allowances (Rydmark et al., 1992) but, for stretching during the flotation step, the connective tissues of the interstitium may stretch more than the contained cells (Deverell et al., 1989). Controls for volume measurement include polystyrene latex particles and beads (Salisbury and Whimster, 1993). Stereological methods of volume estimation are much easier to apply, and may be considerably more accurate, than 3DR. This is an example where confocal reflectance microscopy could be really useful as the processing of the tissue could be followed right through, starting with the living tissue.

3. Surface area measurements

Stereological methods (Briarty, 1975) provide the obvious means for the estimation of surface areas but such measurements can also be made from 3-D models. Care has to be taken, however, because the estimated surface area may vary with the magnification of the model. This is because of the «coastline effect» of greater complexity with increasing magnification (Cross et al., 1994; Salisbury and Whimster, 1994). Measurements of volume from 3DR models are valid, however, as the changes in volume generated by the «coastline effect» are normally very small and they can normally be ignored.

Presentation of images

Single monochrome images look very two-dimensional (which of course they are!). Viewing stereo pairs can be used an as aid to topographical localization in 3DR (e.g. Van Reempts and Borgers, 1994) but many people find they cannot see these as anything other than two images side by side. Presenting «triplets» made up of anterior, posterior and lateral views helps interpretation considerably, as does colour representation (e.g. Deverell et al., 1993; Salisbury and Whimster, 1993; Salisbury et al., 1993). In practice, watching the 3-D image stack being drawn by the computer program on the monitor screen is also very helpful, as is viewing the rotating image on the monitor, where visual cues about depth become apparent from motion parallax. Recording the sequence of successively rotated images on the screen onto videotape can be used to show the 3DR images at conferences (Whimster and Cookson, 1992). Hard discs or rewritable optical storage media as special supplements to journals are possible, albeit still relatively expensive, and some journals are known to have considered this recently. Unauthorised reproduction of data is a problem. The use of concealed fast Fourier transforms to protect data has been suggested but introduces too much noise into the images. In general, researchers are unwilling to supply journals and data bases with information describing large 3-D data sets unless they are convinced it is of no further use of them.

Applications of 3DR in histology

1. Anatomical studies

An increasing variety of investigations have been made in normal anatomy and microanatomy using 3DR. Examples in animals include studies of frog heart (Gras and Killmann, 1983; Grass, 1984), of the larynx of the moustache bat (Johnson and Capowski, 1983) and of pressure-volume relationships in alveoli in rat lung

(Mercer et al., 1987). In humans, Kimura et al. (1977) have investigated the relationship between the enamel and dentine of teeth, and Campbell and others (1992) have reconstructed colonic crypts to examine the localization of specific cell types.

2. Developmental biology

3DR has an obvious use in comparing organs at different stages of development. In human embryos and fetuses, it has been employed to examine the anatomical structure of the cardiovascular and central nervous systems (Deverell et al., 1993; Salisbury and Whimster, 1993) and the distribution of notochordal tissue (Salisbury, 1992; Salisbury et al., 1993). At an ultrastructural level, Dorup and colleagues (1983) studied the developing human proximal renal tubule.

3. Neurosciences

In neurobiology, 3DR can act as an aid to understand both individual cells and the complex functional networks providing communications between cells. Pacek (1971), for example, made 3DR models of astrocytes and oligodendrocytes and Street and Mize (1983) studied the distribution of synapses on a neuronal cell surface. Mercer and others (1987) have examined macular receptive field organization and Rydmark and colleagues (1992) have made 3-D studies of cat cerebral cortex. Afshar and Dykes (1982) reconstructed the human brain stem.

Applications of 3DR in histopathology

Micropathological applications of 3DR have been in both the neoplastic and non-neoplastic fields. Takahashi and his co-workers have published a considerable volume of work on 3-D aspects of gastric neoplasms (Takahashi and Iwama, 1984a,b; Yaegashi and Takahashi, 1990). Chawla and colleagues (1981) have made reconstructions of secondary deposits. 3-D object counting, such as the number of cleaved nuclei in cases of centrocytic lymphoma, is a further use. Non-neoplastic applications have included cirrhotic nodules (Yaegashi et al., 1987) and the biliary pathways in cirrhotic livers (Yamada et al., 1987).

The future for 3DR

As the above account makes clear, 3DR has become a valuable tool for many biological researchers and there is clearly much that has been, and still can be done, with the techniques as they currently stand. However, the development of the methodology for 3DR from serial tissue sections has probably been taken as far as it can go at present.

For 3DR quantitation to become an acceptable tool in routine diagnostic histopathology will necessitate it being speeded up considerably. This has to be by the automatic acquisition of data from a confocal microscope, with direct input into a 3DR software package on a microcomputer. The major problem to be overcome here is the limited depth of sampling in a confocal microscope, even with focus scan memory. The next advances may be in methods of «clearing» tissues, by solvents or other chemicals, so that a greater thickness can be sampled automatically in the z-plane.

High spatial resolution magnetic resonance images (MRI) could also provide a replacement for some of the present 3DR images. MRI scans of single digits, excised tissues e.g. a part of the brain, single objects up to 5 mm in diameter, and parts of larger objects up to 150 mm in diameter have already been performed (Carpenter et al., 1992). The cost of the equipment is very expensive (about £300,000) and the spatial resolution currently attainable (10-20 μm) is not particularly high. MRI scans can, of course, though be performed on living tissues and provide a safe and non-invasive method for studying living animals and humans.

The future of 3-D analysis of biological structures looks bright.

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