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A comparative study of the quantitative accuracy of three-dimensional reconstructions of spinal cord from serial histological sections

B. S. DUERSTOCK, C. L. BAJAJ* & R. B. BORGENS
Center for Paralysis Research, Institute for Applied Neurology, Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, U.S.A.
**Department of Computer Sciences, Center for Computational Visualization, The University of Texas at Austin, Austin, TX 78712, U.S.A.*

Summary

We evaluated the accuracy of estimating the volume of biological soft tissues from their three-dimensional (3D) computer wireframe models, reconstructed from histological data sets obtained from guinea-pig spinal cords. We compared quantification from two methods of three-dimensional surface reconstruction to standard quantitative techniques, Cavalieri method employing planimetry and point counting and Geometric Best-Fitting. This involved measuring a group of spinal cord segments and test objects to evaluate the accuracy of our novel quantification approaches. Once a quantitative methodology was standardized there was no statistical difference in volume measurement of spinal segments between quantification methods. We found that our 3D surface reconstructions' ability to model precisely actual soft tissues provided an accurate volume quantification of complex anatomical structures as standard approaches of Cavalieri estimation and Geometric Best-Fitting. Additionally, 3D reconstruction quantitatively interrogates and three-dimensionally images spinal cord segments and obscured internal pathological features with approximately the same effort required for standard quantification alone.

Introduction

Three-dimensional (3D) computer visualization is useful for imaging biological features, and the same holds for its ability to quantify the reconstructed 3D surfaces (Moriarty *et al.*, 1998). Using 3D surface reconstructions obtained from serial histological sections for quantitative querying is now available to biological scientists. Attempts have been made in the past to measure 3D images after their development or from their component slices. Popular quantification methods for calculating the volume of soft tissues include Geometric Best-Fitting, based on shape assumptions and stereological procedures. The Cavalieri method is a commonly used quantification method for unbiased estimation of the volume of a variety of biological objects from serial histological sections (Mattfeldt, 1987; Michel & Cruz-Orive, 1988; Howard *et al.*, 1993; McNulty *et al.*, 2000). In this study, volume was calculated by multiplying section thickness by the area of interest in the data set of sections, either directly measured through computerized planimetry (Gundersen & Jensen, 1987; Cruz-Orive, 1989, 1999; Mattfeldt, 1989) or estimated using point grids randomly placed on the sections (Cruz-Orive, 1993, 1999; Roberts *et al.*, 1993, 1994). The Geometric Best-Fitting technique compares anatomical structures of interest to known geometric shapes (i.e. an elliptical cylinder or a sphere). Volume estimation dependent on geometric shape is then used to approximate the size of the histological tissue, provided that the geometric holds (Blight, 1985; Noble & Wrathall, 1985; Harris & Stevens, 1988; Bresnahan *et al.*, 1991).

In Moriarty *et al.* (1998) and Duerstock *et al.* (2000) we showed that volume measurements of spinal cord could be quantified from the 3D reconstructed surfaces themselves. We used two non-commercially available 3D surface reconstruction algorithms, referred to as Isocontouring and Surface Tiling, to produce precise wireframe surfaces of biological features of interest that can be accurately measured for their volume (Bajaj *et al.*, 1996a,b, 1997, 1999). We tested the accuracy of these two 3D quantification methods when compared to stereological methods and Geometric Best-Fitting in a morphological study of injured spinal cord segments and their internal pathological structures. However, slight quantitative differences in measurements were observed between Isocontouring and Surface Tiling and the standard quantitative approaches (Duerstock *et al.*, 2000). Thus, a comprehensive evaluation of the accuracy of these 3D reconstruction methods compared to unbiased stereological and model-based quantification methods was performed.

Correspondence to: Dr B. S. Duerstock. Fax: 001 765 494 7605; e-mail: bsd@vet.pudue.edu

Materials and methods

Animals

Adult (300 g) laboratory guinea-pigs were used in these experiments. Following surgery (see below), they were housed two animals per cage, fed *ad libitum*, and their health monitored daily. Ten animals in this study were killed by anaesthetizing them with 0.2 mL of Ketaset and 0.2 mL of Rompun then an overdose of Sodium Pentobarbital (0.8 mL of 1 g mL^{-1} standard injectable) immediately followed by perfusion/fixation with 6% paraformaldehyde, 0.5% glutaraldehyde in a phosphate buffer. Spinal cords from each animal were dissected free and immersion fixed in the above fixative for approximately 18 h.

Histological preparation

A segment of spinal cord from the thoracolumbar region (*c*. 1 cm in length) was cut from each spinal cord and dehydrated in ascending concentrations of alcohol followed by xylene, permitting infiltration and embedding in Paraplast (paraffin) by conventional methods. The 10 spinal cord segments were transversely sectioned on a rotary microtome and affixed to microscope slides. Serial consecutive sections approximately 20 μm thick were sectioned from these spinal cord segments. Prior to use, the slides were dipped in a 0.5% gelatin solution that aids in the adhesion of the sections to the slides during subsequent treatment. Paraffin was partially removed with a 1-h treatment in a 60 °C oven, and completely removed after a 1-h immersion in 100% xylene. Sections were rehydrated by immersions in descending grades of alcohol to distilled water by conventional methods. The sections were stained with neutral red, rinsed in distilled water and coverslipped in permount.

Video capturing and registration of serial sections for 3D visualization

An Optronics DEI-750® (Goleta, CA, U.S.A.) colour video camera mounted on the Olympus Van Ox® (Optical Analysis, Indianapolis, IN, U.S.A.) universal microscope displayed histological sections on a computer monitor. Histological images were acquired to an Intel® dual Pentium (Santa Clara, CA, U.S.A.) computer using Adobe Photoshop® (San Jose, CA, U.S.A.) software and managed on a Silicon Graphics® Indigo (Mountain View, CA, U.S.A.) for 3D reconstruction.

The spinal cord sections were acquired to the computer at low magnification (20×). Ten consecutive histological sections from each spinal cord were captured. During the digitization process, registration was accomplished by superimposing each successive image onto a tracing of the prior histological section on the computer screen by optimally positioning and rotating the microscope stage. The boundaries of the spinal cord, the grey matter and central canal served as effective fiducial markers.

Quantification methods

Samples of 10 consecutive cross-sections were randomly chosen from segments of spinal cord extracted from the thoracolumbar region. Volume estimations were calculated for each sample set from 10 different spinal cord segments to compare quantification between Surface Tiling 3D reconstruction, Isocontouring 3D reconstruction, Cavalieri volume quantification using computerized planimetry and point grids, and Geometric Best-Fitting using shape models. We measured spinal segments using serial transverse sections of similar size so that each quantitative method may be performed.

Surface Tiling Method

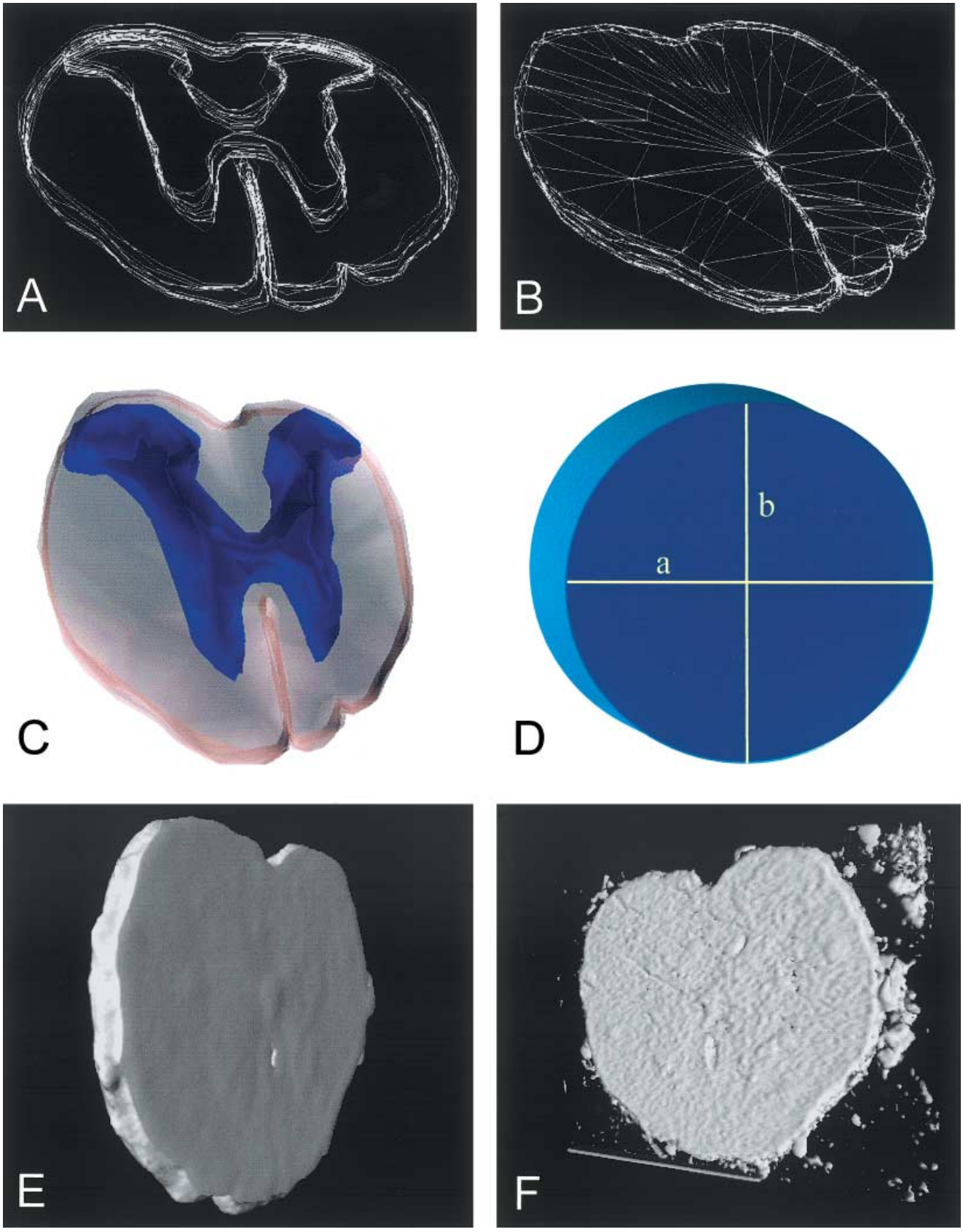
For Surface Tiling reconstruction, we employed the computer program detailed in Bajaj *et al*. (1996b, 1999) to trace contours around the spinal cords on each histological image using a mouse on a Silicon Graphics Indigo™ UNIX workstation (Fig. 1A). The serial contour tracings from the sets of histological sections were used to reconstruct 3D wireframe surfaces of the spinal cord segments, which were then quantified (Fig. 1B).

To estimate volume by the Surface Tiling method, the regions formed between two adjacent contours, defined as a prismatoid, were computed and summed for the entire 3D tiled surface (Bajaj *et al*., 1996a; Moriarty *et al*., 1998) (Fig. 1B). For Isocontouring, a wireframe surface was divided into tetrahedral subcomponents whose volumes were automatically estimated by using a B-spline function (Bajaj *et al*., 1997).

Cavalieri methods

The Cavalieri method was used as the 'gold standard' for stereologically estimating volume (Gundersen & Jensen, 1987; Howard & Reed, 1998). In one approach of the Cavalieri method, subsequently termed 'planimetric approach', the areas of the same sets of spinal cord contours circumscribed during Surface Tiling reconstruction were automatically measured and multiplied by section thickness using a computerized morphometry software program we created (Fig. 1A). Thus, volume was estimated by multiplying the unit area, A, of each spinal cord contour by section thickness, T, and then summing them, $\hat{V} = T \cdot \sum_{i=1}^{m} A_i$. The planimetry and Surface Tiling software program was modified to allow the section thickness or 'Z' distance between sections to be scaled by a user-defined factor. The coefficient of error of the Cavalieri volume estimate of each systematic sample of cross-sections was calculated. The predicted coefficient of error from the samples of spinal cross-sectional areas was 9.3%, 5.6%, 6.6%, 4.1%, 8.2%, 4.7%, 9.4%, 5.6%, 6.5%, and 4.3%, respectively, according to Gundersen & Jensen (1987) equation,

$$CE(\hat{V}) = \frac{1}{\sum A}\left(\frac{1}{12}\{3a + c - 4b\}\right)^{\frac{1}{2}}$$ (refer to Table 3).


A
B
C
D
a
b
E
F

A second approach of the Cavalieri method used grids of points, P, to estimate the volume of the spinal segments, $\hat{V} = T \cdot \frac{a}{p} \cdot \sum_{i=1}^{m} P_i$. a/p is the area of the grid associated with a single point (Howard & Reed, 1998). 'Point counting' was used as a reliable, unbiased approach to confirm the accuracy of volume quantification by 3D reconstruction. The same 10 sets of cross-sections were estimated. NIH Image™ was used to randomly place grid computer overlays over the histological sections for point counting. The predicted error variances during point counting were assumed to be similar to those calculated from planimetric volumes because of the small 'between-section' space of our consecutive sections and their regular shape (Kiêu *et al.*, 1999; García-Fiñana & Cruz-Orive, 2000).

Geometric Best-Fitting

Geometric Best-Fitting was accomplished by assuming that the shape of a spinal cord segment represents an elliptical cylinder (Blight, 1985). Volume was estimated by entering the dimensions of the spinal cord segments into the respective mathematical formulas for an elliptical cylinder, $V = \pi abh$, where a and b are one-half the major and minor axes of the ellipse (spinal cord) and h is the height of the spinal segment (Fig. 1D).

Isocontouring method

The Isocontouring method is a second 3D reconstruction technique that produced 3D surfaces based on the pixel values or 'isovalues' of the component histological sections (Fig. 1E). The algorithmic principles behind Isocontouring reconstruction are detailed in Bajaj *et al.* (1997).

A filter was used on each spinal cord data set to enhance Isocontouring (Fig. 1F). The filter consisted of two steps, convolution and averaging. Convolution reduced noise in an image slice by normalizing the pixel values in each slice. To diminish artefacts and histological defects, the section images were averaged together. Averaging combined overlapping groups of three consecutive section images into a single image. Averaging allowed only biological features that were consistent in two or more histological sections to be three-dimensionally reconstructed (Duerstock *et al.*, 2000).

The only user intervention necessary for reconstruction by Isocontouring was to select the voxel value or isovalue for the 3D surface(s) to be generated. Part of the Isocontouring algorithm is a graphical interface, called the contour spectrum, which is used to select the isovalue. Once the user has selected an isovalue then the software program automatically constructs the 3D isosurface consisting of that value. The contour spectrum displays signature waveforms as B-spline functions of the scalar data set represented as a 3D triangular mesh. The 'gradient integral' is a metric based on the slope or gradient of one of these functions. The usefulness of the gradient integral metric is to find and display prominent surfaces in the data. Specifically, isovalues were selected at peaks in the gradient integral of the contour spectrum for the generation of all 3D reconstructions. This eliminates user subjectivity of selecting 3D features of interest based solely on visual inspection (Bajaj *et al.*, 1997).

Statistical evaluation

Estimates of the spinal cord segments according to the aforementioned methods were compared using a paired, two-tailed Wilcoxon (non-parametric) test for significance. Computations were performed using InStat® (GraphPad Software, San Diego, CA, U.S.A.) software.

Results

Quantification of test objects

We compared the volumes of test objects estimated by 3D reconstruction methods, Isocontouring and Surface Tiling, to Cavalieri estimation methods and Geometric Best-Fitting. We three-dimensionally reconstructed test wireframe models of known size to judge their quantitative accuracy. A rectilinear box was three-dimensionally reconstructed from three sections each containing a square of identical size (Fig. 2A). The volume of the box was calculated by 2D planimetry or by $width \times depth \times height$.

Both Isocontouring and Surface Tiling methods calculated a smaller volume for the test box to stereological methods (Table 1). This was attributed to the way the wireframe models were constructed (Fig. 2, columns B and C, top). As shown in Fig. 2, column B, when Surface Tiling reconstructed the box from three serial sections, it enclosed the wireframe surface by adding pyramids to cap the ends of the box. Isocontouring added flat ends to cap the box; however, these caps are only a fraction of the full section thickness (Fig. 2, column C). Both

Fig. 1. Three-dimensional surface reconstruction and quantification of a segment of spinal cord. During Surface Tiling, contours were traced around the spinal cord in 10 consecutive transverse histological sections. A shows the 10 contours of one of the spinal cord segments stacked on top of each other in registration. During 3D reconstruction, the contours are tiled by triangular primitives to produce the wireframe surface in B. In C, the same spinal segment data set as in A and B is shown with a surface rendering. The transverse plane of the brown spinal segment is facing the viewer with the internal grey matter shown in blue. Note the ventral fissure of the spinal cord at the bottom of the image. The Geometric Best-Fitting method estimates the size of a spinal segment by comparing it to an elliptical cylinder (D). a and b are the major and minor axes of the ellipse used to calculate the cross-sectional area of the spinal cord. E shows the same spinal segment in A–C three-dimensionally reconstructed using the Isocontouring method. The spinal segment is rotated 180° in the horizontal plane. F shows the spinal segment in E without any filter applied. Without filtering the 3D surface becomes noisy and lacks definition.

Table 1. Quantitative querying of a test box from serial sections by different quantification methods. A test box comprising three identical sections was quantified using Cavalieri estimation methods (planimetry and point counting) and 3D reconstructed and measured by Isocontouring and Surface Tiling. The Isocontouring and Surface Tiling methods measured the space between Sections 1 and 3 plus the end caps for the test box in Fig. 2. Therefore, their volume measurements were slightly greater than the exact measurements shown in column 3. Planimetry and point counting measured the thickness of every section from 0 to 3. For sections of equal size, planimetry measures volume exactly.

	Isocontouring	Surface Tiling	Box Size between Sections 1 and 3	Planimetry for Sections 0–3	Point Counting
Volume (mm^3)	0.98	0.81	0.69	1.035	1.05

Surface Tiling and Isocontouring measured the space between sections 1 and 3 plus additional volumes enclosed by the two end caps. This contrasts stereological methods that measure the thickness of each section, thus including the space between sections 0 and 3 (Fig. 2A). Therefore, the box measured by the two stereological methods encompassed a greater volume than the boxes reconstructed by Surface Tiling and Isocontouring. When the cap volumes were removed from the Isocontoured and Surface Tiled boxes and the volume of a single full-thickness section was added, the volume measurements then equalled 1.035 mm^3 – the exact volume of the box created from sections 0–3 (Table 1, column 4).

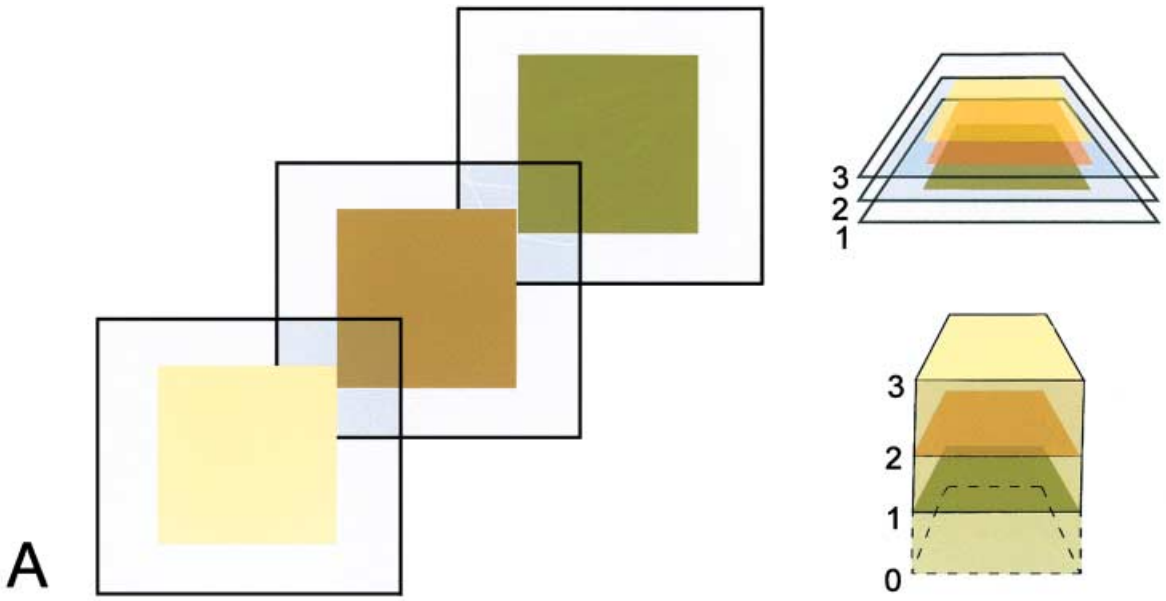
When serial sections are the same size and shape, such as the test box, then planimetry is exact (see Table 1, column 4). However, the precision of the planimetric approach failed when quantifying non-uniform objects from thick sections or with large Z distances between consecutive sections. Figure 3(A) shows another test object, which is an oblate spheroid composed of seven serial sections containing non-uniform sized circles. The test object was measured by each of the different quantification methods, including using the mathematical formula of an oblate spheroid to calculate volume precisely. The volume measurements of the spheroid test object between the different quantification methods were equivalent but much less than from geometric spheroid calculations (Table 2). The test object had few sections and the *Z* distance between sections was greater in relation to the *X* and *Y* dimensions of the sections (Fig. 3). Therefore, large portions of the total spheroid volume could not be explicitly interrogated from the set of serial sections. However, the accuracy of estimating volume between the different quantification methods was similar (Table 2). Slight volume differences between Isocontouring and Surface Tiling were attributed to the manner in which the wireframe surfaces of the spherical test object were constructed between adjacent sections. Because of the large Z distances between consecutive slices, the wireframe surface sloped inward on the Isocontoured spheroid (Fig. 3C), while Surface Tiling spanned triangle primitives from the edge of one contour to the edge of the next, independent of the size differences between consecutive contours (Fig. 3B).

Accuracy of quantitative methods for soft tissues

Three-dimensionally reconstructing and quantifying complex serial histological images with multiple intensity values is intrinsically more difficult than monochrome, symmetrical geometric shapes. Ten spinal cord segments (*c.* 0.2 mm long) were independently measured by Isocontouring, Surface Tiling, and by the three standard quantitative approaches. Table 3 shows the results of the comparison of quantitative approaches between these quantification methods.

When comparing Isocontouring to Surface Tiling, the volume measurements of the spinal segments were not significantly different ($P = 0.75$, paired, Wilcoxon two-tailed test). Also, spinal segment volumes were not significantly different between Cavalieri estimation based on planimetry or point counting and Geometric Best-Fitting methods ($P > 0.05$, Wilcoxon two-tailed test). However, the volume measurements calculated by the 3D surface reconstruction methods (Isocontouring and Surface Tiling) were significantly different from the standard quantification approaches (stereology and Geometric Best-Fitting) ($P < 0.05$, Wilcoxon two-tailed test).

Fig. 2. Three-dimensional surface reconstruction of a test box. In A, three identical sections with coloured squares on a white background were used to three-dimensionally reconstruct a box. The sections are labelled 1–3. For planimetry the area of the square times the section thickness for each section calculated volume, so the box occupies sections 0–3 or $V = T \cdot \sum_{i=1}^{3} A_i$ where T is section thickness and A equals area of the square. The box shown at the top of column B was three-dimensionally reconstructed by the Surface Tiling method. This 3D reconstruction is shown as a wireframe model in which the component sections (1, 2, 3) were stacked vertically. At the top and bottom of the image, pyramidal caps were used to enclose the box during surface reconstruction. When performing Surface Tiling the caps come to a point, which resulted in a smaller volume than Cavalieri estimation by planimetry and point counting (refer to Table 1). Column C shows a 3D reconstruction from the same sections as in A by the Isocontouring method. Thin top and bottom caps were used to cover the wireframe box. These algorithmic differences resulted in smaller computed boxes than those measured by standard quantification methods (Table 1). The bottom of column B shows the surface rendering of the wireframe test box above produced by Surface Tiling. The bottom of column C shows the Isocontoured test box with an opaque surface.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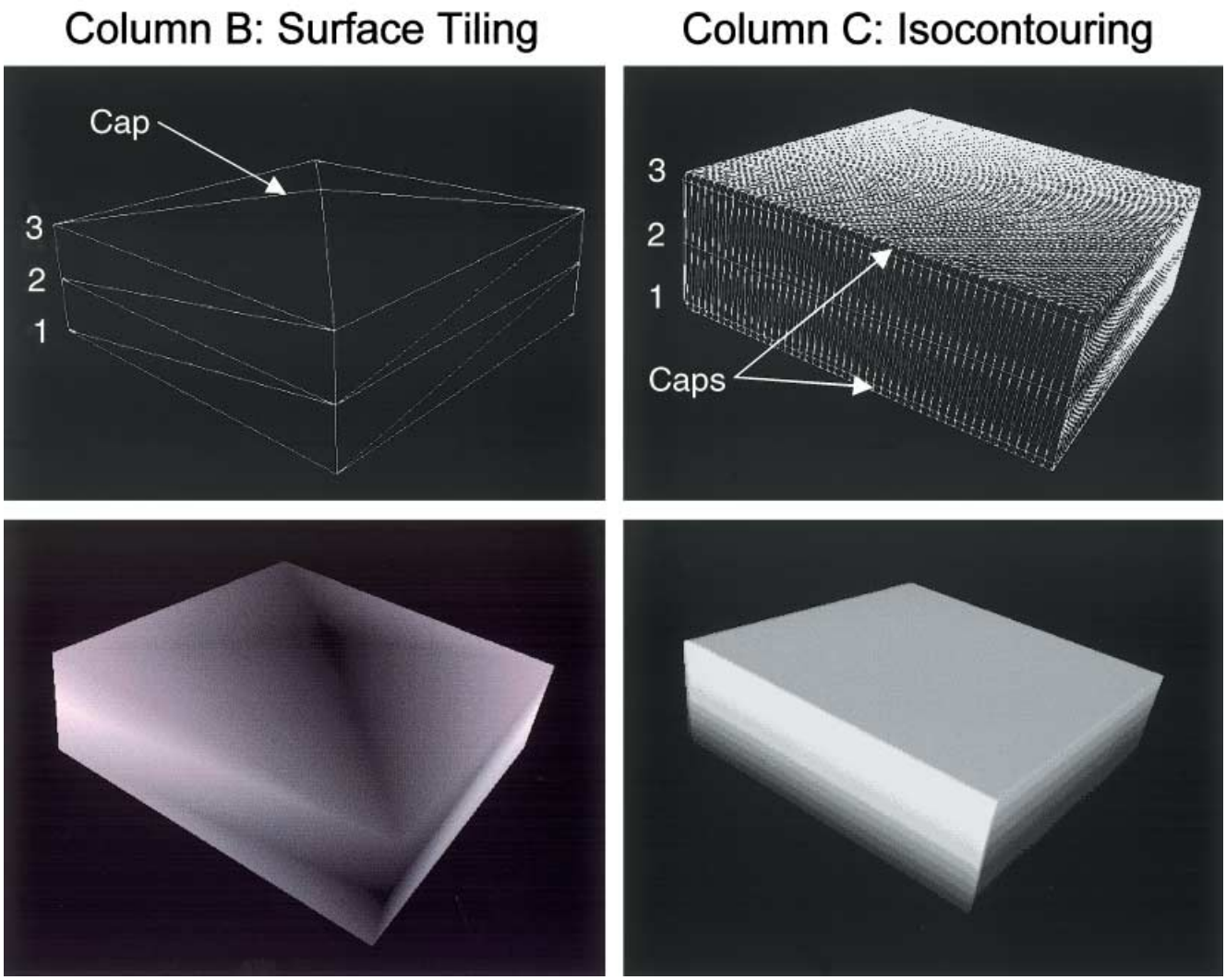
Column B: Surface Tiling
Column C: Isocontouring
Cap
3
2
1
3
2
1
Caps

Table 2. Quantitative querying of a test spheroid from serial sections by different quantification methods. A test spheroid comprising seven concentric circles was quantified using Cavalieri estimation methods (planimetry and point counting) and 3D reconstructed and measured by Isocontouring and Surface Tiling. Although the quantification methods used different approaches, volume measurements were similar. Volume formulas for an oblate spheroid were used to calculate true measurements.

	Isocontouring	Surface Tiling	Planimetry	Point Counting	Spheroid Eqns
Volume (mm^3)	0.91	0.85	0.89	0.85	1.30

Table 3. Comparison of 3D surface reconstruction methods to standard quantification techniques measuring spinal cord segments. Results of volume measurements of 10 spinal cord segments (*c.* 200 μm long) calculated by Isocontouring, Surface Tiling, Cavalieri estimation by planimetry and point counting, and geometric best-fitting. The mean and standard deviation for each group of measurements are provided below. Volume measurements between Isocontouring and Surface Tiling were not significantly different ($P = 0.75$, paired, Wilcoxon). Volume measurements between planimetry, point counting and geometric best-fitting were not significantly different ($P > 0.05$, paired, Wilcoxon). However, volume measurements between the 3D reconstruction methods and standard quantification methods were significantly different ($P < 0.05$, paired, Wilcoxon).

Spinal Segment No.	Isocontouring Cord Volume (mm^3)	Surface Tiling Cord Volume (mm^3)	Planimetry Cord Volume (mm^3)	Point Counting Cord Volume (mm^3)	Geometric Best-fit Cord Volume (mm^3)
1	1.2	1.2	1.3	1.3	1.4
2	1.4	1.4	1.6	1.5	1.6
3	1.1	1.1	1.2	1.2	1.4
4	1.5	1.4	1.5	1.5	1.5
5	1.4	1.4	1.5	1.5	1.6
6	1.5	1.5	1.6	1.6	1.5
7	1.1	1.2	1.3	1.3	1.3
8	1.4	1.4	1.5	1.4	1.6
9	1.6	1.6	1.7	1.8	1.8
10	1.8	1.7	1.8	1.7	1.8
Mean	1.40	1.39	1.50	1.48	1.55
SD	0.23	0.19	0.19	0.18	0.17

Cavalieri estimation by planimetry

From the test objects of known size, it was discovered that significant differences in volume measurements between the 3D reconstruction methods and the standard quantification methods was attributed to calculating the volume between the first and last sections vs. measuring the thickness of every section (see above). This resulted in statistically significant differences in the volume measurements of the spinal cord segments between Cavalieri estimation by planimetry and Isocontouring and Surface Tiling (Table 3). However, when the volumes calculated by planimetry were standardized to compute the space between the first and last sections, the mean volume equalled 1.36 mm^3 (Table 4). Consequently, there was no significant difference between the planimetric approach and Surface Tiling ($\bar{X} = 1.39\ mm^3$) or Isocontouring ($\bar{X} = 1.40\ mm^3$) ($P > 0.05$, Wilcoxon two-tailed test) after standardization (Table 4).

Fig. 3. Three-dimensional surface reconstruction of a spheroid test object. In A, seven sections with concentric circles that increased and then decreased in size were used to three-dimensionally reconstruct a spheroid test object. The sections are labelled 1–7. For Cavalieri estimation by planimetry and point counting, the area of the circle or number of points within the circle, respectively, multiplied by section thickness of each section was used to calculate volume. In B, the spheroid object was three-dimensionally reconstructed by Surface Tiling from the seven sections shown in A. For the wireframe surface, triangle primitives spanned from the edge of one contour to the edge of the adjacent contour. The first and last sections are at the top and bottom of the image. In C, the seven sections are three-dimensionally reconstructed by the Isocontouring Method. The first and last sections are at the top and bottom of the image and at the same angle as in B. However, tessellation between Surface Tiling and Isocontouring was different. With Isocontouring, the triangle primitives sloped inward between consecutive contours to produce a stair-like wireframe surface. Note, however, the volume of the spheroid between quantification methods was similar (Table 2).

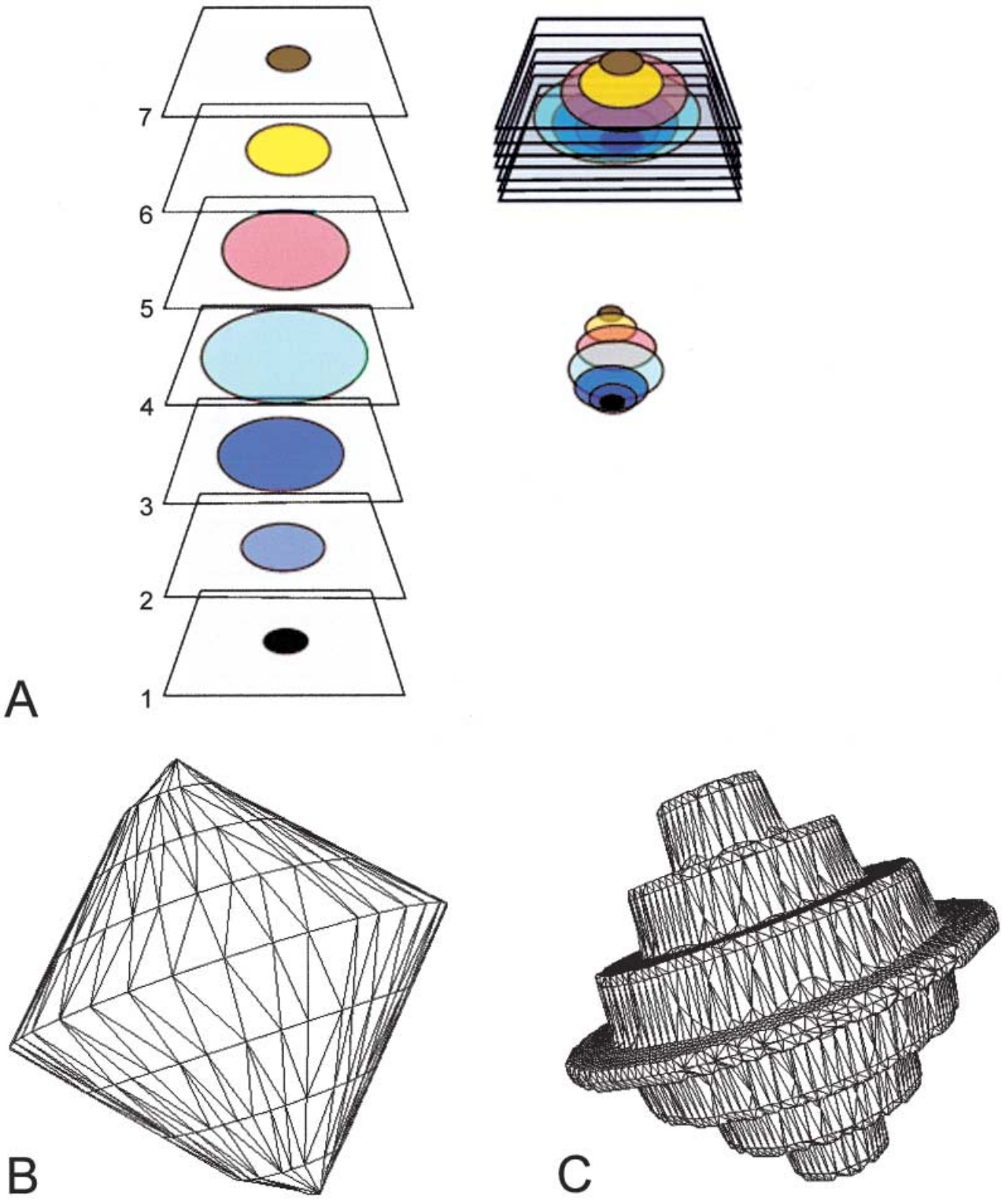
7
6
5
4
3
2
1
A
B
C

Table 4. Standardization of quantitative querying between the first and last sections of spinal cord. Quantification of the 10 spinal segments by each of the quantification methods was standardized by measuring between histological sections 1 and 10. The calculation of spinal segment volume between each of the quantification methods was not significantly different ($P \geq 0.05$, paired Wilcoxon two-tailed test) except between Surface Tiling and point counting, which resulted in comparable numbers but statistically different ($P = 0.004$, Wilcoxon).

	Isocontouring Cord Volume (mm^3)	Surface Tiling Cord Volume (mm^3)	Planimetry Cord Volume (mm^3)	Point Counting Cord Volume (mm^3)	Geometric Best-fit Cord Volume (mm^3)
Mean	1.40	1.39	1.36	1.34	1.40
SD	0.23	0.19	0.17	0.16	0.15

Cavalieri estimation by point counting

Unbiased volume measurements of the spinal segments were calculated by applying point grids to serial sections. The stereological approach also interrogated the thickness of every section while Isocontouring and Surface Tiling only measured the volume between the first and last sections plus the end caps. Stereological volume measurements in Table 3 were standardized to measure only the space between sections 1 and 10 for the spinal cord data sets (Table 4). The stereological mean volume then became 1.34 mm^3 and was not significantly different from Isocontouring volume measurements ($P = 0.25$, Wilcoxon two-tailed test). Likewise, when Isocontouring volume measurements were standardized to calculate the thickness of all 10 sections of the spinal cord data sets, the mean volume became 1.42 mm^3, which was not significantly different from the stereological volume measurements in Table 3 ($P = 0.11$, Wilcoxon two-tailed test). Therefore, the inclusion or exclusion of a single section from very small data sets of few sections will significantly impact quantification methods, as one might expect.

Geometric Best-Fitting

Geometric Best-Fitting has been shown to be satisfactory in estimating the volume of segments of spinal cord using standard equations for elliptical cylinders (Blight, 1985; Moriarty *et al.*, 1998; Duerstock *et al.*, 2000). The original volume measurements calculated by Geometric Best-Fitting were larger than those from other quantification techniques but within statistical significance to Cavalieri estimation by point counting or planimetry ($P > 0.05$, Wilcoxon two-tailed test) (Table 3). However, when Geometric Best-Fitting calculated only the region between the first and last sections, similar to the Isocontouring and Surface Tiling Methods, then the mean volume equalled 1.40 mm^3, yielding no significant differences between quantification methods ($P > 0.05$, Wilcoxon two-tailed test) (Table 4).

Discussion

In this study we investigated the accuracy of various methods of 3D quantification using a large sample of spinal cord segments and by evaluating test objects. We concluded that direct quantification of 3D reconstructed surfaces is a valid means of measuring the volume of spinal cord tissues when stereological methods are practised, such as understanding the algorithmic principles behind the type of 3D reconstruction method being used and systematic sampling. Quantitative differences between Surface Tiling and Isocontouring methods and stereological approaches were caused by fundamental differences in how wireframe models are constructed, not by inherent inaccuracies in the quantitative querying of 3D reconstructed surfaces. After standardizing quantitative interrogation between the first and last sections of a data set, 3D reconstruction provided statistically similar volume estimates according to Cavalieri estimation by planimetry or point counting and Geometric Best-Fitting. For small data sets the omission of a single section resulted in substantial quantitative differences; however, in Duerstock *et al.* (2000) it was demonstrated that the exclusion of a single section in large reconstructed spinal cord data sets did not significantly affect volume measurements calculated by 3D reconstruction compared with Geometric Best-Fitting and Cavalieri estimation by planimetry.

The question was not whether volume could be accurately computed from 3D reconstructed wireframe surfaces but whether these surfaces were accurate representations of the actual tissue samples. Thus, the precision of the 3D models needed to be determined. Unfortunately, 'true' volume measurements of spinal segments could not be calculated for comparison with our 3D quantification methods. Measurement techniques, such as the Archimedean water immersion test, could not precisely measure these minute tissues. Furthermore, measurements of intact spinal cord segments cannot be compared to spinal segments from a series of histological sections. It has been estimated that shrinkage by fixation, embedding and sectioning during histological preparation would decrease the amount of tissue by up to 8% (Blight & Decrescito, 1986; Deverell *et al.*, 1993). Therefore, 3D reconstructing test objects of known size to evaluate the accuracy of these quantitative methods was performed.

Cavalieri planimetric method

For symmetrical structures, Cavalieri estimation by planimetry provided comparable quantitative analysis to our 3D

reconstruction methods. When we measured cylindrical spinal cord segments from transverse sections that maintained a uniform shape and size, volume measurements were similar (Table 4). However, there were definite inaccuracies using the planimetric approach when interrogating complex 3D structures where contours change shape and size from one section to the next for thick systematic slices. Error variance in the planimetric approach is small only if the volumetric change from one section to the next is nominal (Salisbury, 1994; García-Fiñana & Cruz-Orive, 2000). Since 3D reconstruction spans triangular primitives from the edge of one contour to the edge of the next, it is more flexible in the kinds of shapes that can be modelled and measured (Fig. 3).

In conclusion, tiling by 3D reconstruction models the natural character of biological structures – no matter how complex the topology – more accurately than the slab-stacking technique executed by planimetry. More in-depth study is needed to evaluate the effect on quantification when 3D surface tiling extremely complex structures, particularly those involving branching structures, such as blood vessels, dissimilar contours and under-sampled data sets.

Geometric Best-Fitting

Although Geometric Best-Fitting is not used for accurate quantification, we have shown that when anatomical structures closely resemble geometric shapes, such as spheres, cylinders and ellipsoids, then this method could reliably measure volume. Geometric Best-Fitting does not faithfully reproduce the actual morphology of the specimens being interrogated (see the spinal segment in Fig. 1D). Therefore, the precision of Geometric Best-Fitting for quantifying volume is extremely limited, particularly for complex morphologies.

Three-dimensional surface reconstruction

Surface Tiling and Isocontouring each employ different algorithmic methods but volume measurements were statistically similar. For instance, during the Surface Tiling method, the user manually selects and traces contours around biological features of interest for each histological section. For the Isocontouring method, the user relies upon differences in pixel intensity within the histological images to select structures of interest aided by a contour spectrum to select significant isosurfaces.

To maintain quantitative accuracy we used every consecutive histological section for 3D reconstruction instead of choosing every other section or multiples of sections. Therefore, histological artefacts and defects in the sections, such as folds and tears, poor colour contrast staining within histological images, and correspondence and branching problems between adjacent contours affect the quality of 3D reconstructions of biological specimens (Bajaj *et al.*, 1996b; Duerstock *et al.*, 2000).

During Surface Tiling minor histological defects can be ignored by the user when contour tracing. However, since surface extraction during Isocontouring is based upon pixel intensity differences within the histological sample, it has been necessary to use a filter to reduce the noise and artefacts present in the histological slices (Duerstock *et al.*, 2000). As illustrated in Fig. 1(E,F), filtering helped produce distinct and clear 3D reconstructions that were necessary for accurate quantification. However, we determined that filtering a data set of slices could affect quantification.

We evaluated the effects of filtration on quantification by measuring Isocontoured histological sections from the 10 spinal cord segments at multiple filter iterations. Each data set required differing amounts of filtering depending upon the colour contrast and noise of the sections. The effects of filtering varied according to the sample. In general, when filtration was drastically increased then the shape of a structure of interest became less complex and more spherical, resulting, in some cases, in a slightly larger volume measurement. However, using no or low filtration resulted in 3D images that were too noisy and undefined causing erroneous volume measurements (Fig. 1F). The proper amount of filtration varied according to the quality of the histological sections in the data set.

The size of the triangle primitives used during tessellation of wireframe surfaces may also affect the smoothness of shapes. As shown in Figs 2 and 3, Isocontouring used triangles that are much smaller than those used for Surface Tiling. Smaller surface primitives produced smoother, rounded 3D surfaces that perhaps resulted in slightly increased volume measurements during Isocontouring. Owing to smaller primitive size, Isocontouring produced smoother 3D surfaces than Surface Tiling, which characterized the natural texture of spinal cord tissue more realistically. Further investigation is needed to determine how differences in primitive size can affect overall quantification between different 3D surface reconstruction methods. We believe that the smaller the primitive, the more closely will the 3D surface model the actual tissue.

Cavalieri point counting

The accuracy of measuring the volume by 3D reconstruction was further validated by the Cavalieri method. The point counting method employs an unbiased stereological approach that has historically served as an accurate and efficient procedure for volume quantification. The point counting procedure is possibly more efficient than Cavalieri estimation based on planimetry in one important way. It is easier to decide whether grid points lie on an object than manually circumscribing objects of interest on each section (Gundersen *et al.*, 1981; Howard & Reed, 1998). We did not determine whether this affected quantitative accuracy.

We do not propose that quantitative querying of three-dimensionally reconstructed wireframe surfaces would replace the accuracy of Cavalieri approaches. We assert, however, that accurate measurements from 3D reconstructions can be made without human interaction when unbiased and systematic

methods are used. We used all small thickness histological sections through the spinal cord segment for quantification, even for very large data sets (Duerstock *et al.*, 2000). This protects against sampling bias and imprecise measurement of complex features present in only a few consecutive sections. Advancements in 3D imaging technology have made the effort required to produce 3D reconstructions not much greater than stereological procedures. In addition, further morphological information can be gathered from the development of 3D images and through virtual dynamic navigation within the reconstructed biological tissues.

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