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THE EFFECT OF IMPROPER REALIGNMENT OF SERIAL SECTIONS ON SURFACE AREA MEASUREMENTS OF 3D – RECONSTRUCTED OBJECTS

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ABSTRACT

Stereological estimation of surface area of three-dimensional objects using serial sections usually requires perfect realignment of the sections. In physical sectioning often fiducial markers are used to realign the sections. Nevertheless shrinking and other deformations of the slices frequently make it impossible to obtain a precise reconstruction of the spatial arrangement. This inaccuracy causes an error in surface area estimation using methods as e.g. the spatial grid. As shown in the paper, it leads to an overestimation of the surface area. The error is related to the deviation of the "true" slice positions and a simple method is suggested to calculate an upper bound of the error by comparing the relative positions of the fiducial marker profiles on the sections. This method is successfully applied to the estimation of a fish brain's surface area.

Key words: 3D-reconstruction, section realignment, spatial grid, surface area.

INTRODUCTION

A well-known problem — not only to life scientists — is how to estimate total surface area of particles when only a few specimen are available. Popular solutions consist in evaluation of serial section stacks by means of three-dimensional reconstruction or equivalent stereological methods as the spatial grid (Sandau, 1987).

In contrast to volume determination following the Cavalieri principle, surface area measurements require a perfect realignment of the sections. This is no problem if optical sections are made, as demonstrated by Howard and Sandau (1992) for the CSLM. However, most often there is no choice but physical sectioning. Here, realignment is rendered difficult by uncontrollable shrinkage and stretching of the slices.

A lot of studies, e.g. Laan et al. (1989) and Rydmark et al. (1992), have focussed on restoring the original position of sections. Several proposed methods require computer assistance or digital image processing and are based on rigorous assumptions about the quality of slice deformation. A common practice is the use of fiducial markers parallel to the section axis. They may consist in embedded material (e.g. Johnson et al., 1989) or drill holes (e.g. McLean et al., 1987).

If the sections are not accurately realigned, the reconstructed surface appears jagged and folded, see Fig. 1. Folding, however, is a well-known principle of surface area increase!

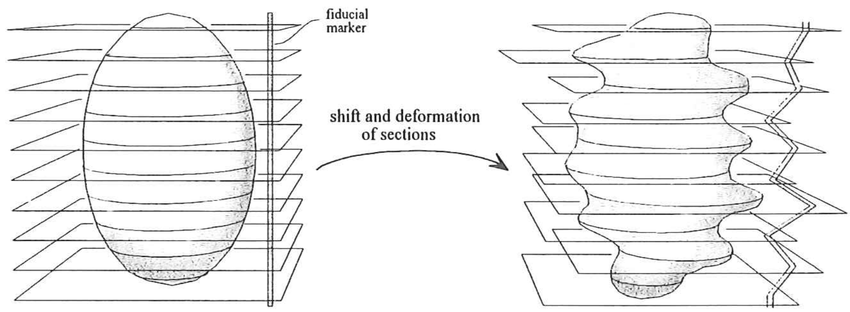

Fig. 1. Improper realignment and deformation of sections lead to seeming surface area increase.

The present paper deals with an assessment of the amount of this increase. Oblique sectioning ensues a similar overestimation of surface area, which is shortly discussed after the derivation of a bias formula. In the following section, the estimation of the surface area of a fish brain's part (tectum opticum) is given as a practical example. Suggestions how to apply the theoretical findings can be found in the concluding section.

DERIVATION OF A BIAS FORMULA

A theoretical experiment shall be carried out to asses the bias:

The apparent, biased surface area $S_{apparent}$ is estimated with the help of a spatial grid (Sandau, 1987). The expectation $\mathbb{E}(\hat{S}_{apparent})$ of this estimate is then compared with the true surface area S_{true} of the object. The section axis is supposed to have been chosen randomly.

The spatial grid method uses three mutually perpendicular grids of test lines, see Fig. 2. Their directions shall be denoted by x, y and z, and I_x, I_y, I_z shall denote the number of intersections with the surface of interest. With grid spacings δ_x, δ_y and δ_z,

$$\hat{S} = \frac{2}{3} \cdot (\delta_y \delta_z I_x + \delta_x \delta_z I_y + \delta_x \delta_y I_z) \tag{1}$$

is an unbiased estimator of the surface area, provided that direction and position of the grid are suitably randomized. But even in case of restricted randomization, the resulting estimates are rather precise, because the variance due to the direction is small (Hahn and Sandau, 1989, Sandau and Hahn, 1994). In the above mentioned theoretical experiment, it therefore seems admissible to take the sectioning axis as the z-direction of the grid. Then, δ_z just becomes the sectioning distance.

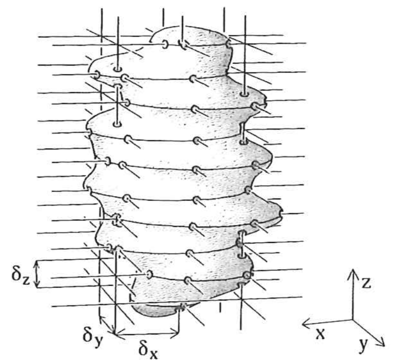

Fig. 2. The apparent surface area is estimated using a spatial grid with spacings δ_x, δ_y and δ_z.

In order to express the estimated area in terms of the true surface area, each section's coordinates are transformed such that the original positions of the object profiles are restored. This transformation of course also alters the grid coordinates, but the numbers of intersections remain unchanged, since intersections and comparable topological properties are not influenced, cf. Fig. 3.

To calculate the mean value $\mathbb{E}(\hat{S}_{apparent})$ of the estimated surface, the expected numbers $\mathbb{E}(I_x)$, $\mathbb{E}(I_y)$ and $\mathbb{E}(I_z)$ have to be known. The expected number $\mathbb{E}(I)$ of intersections of surface of area S with any randomly orientated line system of length density L_V is given by

$$\mathbb{E}(I) = \frac{1}{2} S \cdot L_V. \tag{2}$$

Unfortunately, $S_{apparent}$ depends on the orientation of the sectioning axis. Therefore, Eq. 2 is only applicable to the true surface, that is in the situation of restored original section positions.

For simplicity, only the case of purely horizontal translation of the sections is treated in the following. Obviously, the length densities L_{V_x} and L_{V_y} of the x- and y-grid, respectively, are not affected by horizontal shifts perpendicular to the z-axis. However, L_{V_z} is increased, because the distance between corresponding grid points increases, which just equals the length of the test lines of the z-grid between two adjacent sections, see Fig. 3.

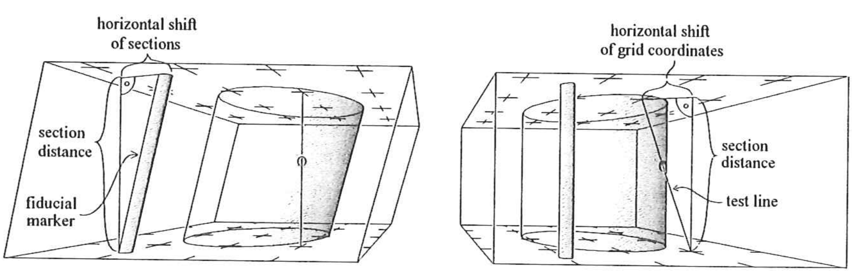

Fig. 3. If improper realignment because of horizontal shifts (left picture) is reversed to restore the original position (right picture), then the length of test lines between two sections increases.

For the uncorrected situation of the sections, this length is equal to the section distance, whilst it amounts $\sqrt{(\text{section distance})^2 + (\text{horizontal shift})^2}$ after transformation to original position. Since the number of test lines between two sections remains unchanged, the corresponding length density L_{V_z} changes from $L_{V_z \text{apparent}}$ in the uncorrected situation to $L_{V_z \text{corrected}}$ after restoring the original positions, where

$$L_{V_z \text{corrected}} = \sqrt{1 + \left(\frac{\text{horizontal shift}}{\text{section distance}}\right)^2} \cdot L_{V_z \text{apparent}}. \tag{3}$$

As already mentioned above, the numbers of intersections are not affected by the transformation. Their expectations,

$$\mathbb{E}(I_x) = \tfrac{1}{2} S_{\text{true}} \cdot L_{V_x},\ \mathbb{E}(I_y) = \tfrac{1}{2} S_{\text{true}} \cdot L_{V_y}, \text{ and } \mathbb{E}(I_z) = \tfrac{1}{2} S_{\text{true}} \cdot L_{V_{z \text{corrected}}}, \tag{4}$$

are introduced in Eq. 1, which to this end is transformed to

$$\hat{S}_{\text{apparent}} = \tfrac{2}{3} \cdot \left(\frac{I_x}{L_{V_x}} + \frac{I_y}{L_{V_y}} + \frac{I_z}{L_{V_z \text{apparent}}} \right). \tag{5}$$

This yields a bias formula:

$$\frac{\mathbb{E}(\hat{S}_{\text{apparent}}) - S_{\text{true}}}{S_{\text{true}}} = \tfrac{1}{3}\left(\sqrt{1 + \left(\frac{\text{horizontal shift}}{\text{section distance}}\right)^2} - 1 \right). \tag{6}$$

Applied to the whole stack of equidistant sections, Eq. 6 turns to

$$\frac{\mathbb{E}(\hat{S}_{\text{apparent}}) - S_{\text{true}}}{S_{\text{true}}} = \tfrac{1}{3}\left(\frac{\sum_{\text{all slices}} \sqrt{1 + \left(\frac{\text{horizontal shift}}{\text{section distance}}\right)^2}}{\text{number of slices}} - 1 \right). \tag{7}$$

In practice, the relative horizontal shift of two consecutive sections can be measured comparing the coordinates of corresponding fiducial marker profiles.

EFFECT OF OBLIQUE SECTIONING

The considerations to assess the bias due to horizontal aberration of section positions can be extended to the case of oblique sectioning:

Let $\alpha < \frac{\pi}{2}$ be the angle formed by the sectioning axis and the direction of the fiducial markers. If the sections are as usual realigned superimposing the marker profiles of adjacent sections, an actual horizontal shift of section positions results. It amounts (horizontal shift) $= \tan\alpha \cdot$ (section distance), which implies a mean bias of

$$\frac{S_{\text{apparent}} - S_{\text{true}}}{S_{\text{true}}} = \tfrac{1}{3}\left(\frac{1}{\cos\alpha} - 1 \right). \tag{8}$$

Here, the bias is independent of the section distance. Normally, obliquity of sectioning can be hold in a moderate range, so that the resulting bias remains small. Eq. 8 shows, that e.g. an obliquity of 10° yields a nearly negligible bias of only about 0.5 %.

APPLICATION

The surface area of the optical tectum – a part of the brain – of a fish (*Oreochromis*) larva was estimated using the spatial grid. To this end, glycol methacrylate (Historesin®) embedded larvae were serially sectioned. Three human hairs were embedded together with the fish larva to serve as fiducial markers.

A stack of slices of 3 μm thickness and with a distance of 18 μm was evaluated. The whole stack comprised 38 sections. Larger section distances of $n \cdot 18$ μm were obtained through fractions of the stack containing every n-th section.

It can be observed that the estimated area essentially depends on the section distance, as long as the distance is smaller than about 90 μm.

Bias was estimated out of the distances of marker traces in adjacent sections following Eq. 7. As Fig. 4 shows, e.g. a bias of about 40 % is expected for a section distance of 18 μm. After correction by the estimated bias, the strong dependence of the section distance could be diminished, and the area estimates stabilized, see Fig. 5.

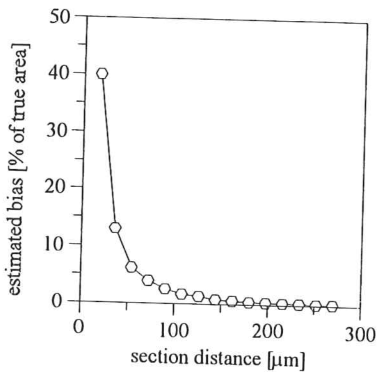

Fig.4. Estimated bias of surface area estimation, in % of true area.

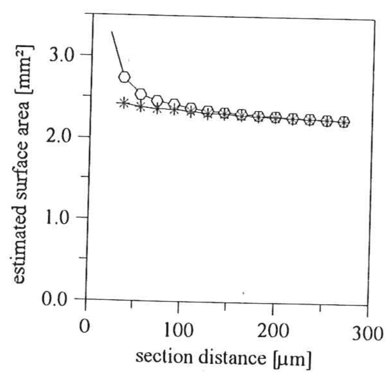

Fig. 5. Estimated surface area, without (-○-) and with (-✳-) correction by bias.

CONCLUSIONS

As the above considerations have shown, biased surface area estimates ensue from improper realignment of serial sections. The amount of bias depends on the ratio of horizontal aberration of section position to section distance. Some examples are given in Tbl. 1, which were calculated using Eq. 6.

Table 1. Bias depending on ratio aberration : section distance

$\frac{\text{aberration}}{\text{section distance}}$	3.0	2.0	1.0	0.5	0.25
resulting bias % of true surface area	72 %	41 %	14 %	4 %	1 %

If a precise reconstruction of the section positions is not possible, it is therefore recommended to chose the section distance large enough to avoid high biases. To this end, Tbl. 1 or Eq. 6 may be used assuming an amount of horizontal aberration obtained from preliminary estimation by comparing marker profile positions or just according to experience.

If the sections are not only translated but also rotated or deformed, then the bias estimate depends on the position of the markers in the sections. Supposed that all markers were used for realignment, the smallest horizontal aberrations of corresponding positions in adjacent sections should occur in the region between the markers. That means that using Eq. 7 together with the horizontal distances of marker traces in adjacent sections yields an upper bound of the actual bias, if the markers are situated outside the object.

Enlarging section distance is of course restricted by surface curvature: if sections are too far apart from one another, curves of the surface are neglected, what on the other hand leads to an underestimation of area. Therefore, the choice of section distance should be done very carefully respecting both effects. On th„e other hand, a larger section distance also means a larger variance of the estimates (Hahn and Sandau, 1989, Sandau and Hahn, 1994). It is usually aimed to minimize the mean square error, $\mathrm{MSE} = \mathrm{variance} + \mathrm{bias}^2$.

Averaging surface area estimates over several fractions of a section stack with section distance being a multiple of the original one may yield better results than evaluating the whole stack all at once.

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