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Correcting Errors in Estimating Neuron Area Caused by the Position of the Nucleolus

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ABSTRACT
The technique of measuring soma cross-sectional area at the plane of the nucleolus leads to systematic errors that depend on how far the nucleolus is displaced from the center of the soma. A set of correction factors was produced based on calculations from a geometric model of the measurement process. Applying the correction factors to measurements of second-order auditory neurons led to substantial changes in the estimated soma area.

Key words: geometric model, eccentricity, neuron size, measurements

Soma area is frequently measured to assess one aspect of morphological change during development or following experimental manipulations (Cowan, '70; Lieberman, '71; Globus, '75; Grafstein, '75). Stereological methods are available that permit the accurate estimation of neuron cross-sectional area or volume (Underwood, '70; Weibel, '79). These techniques are not routinely employed, however, as they require the measurement of a large sample of all elements in a population regardless of how the neurons are sectioned. In many regions of the nervous system, it is impossible to separate the population of interest from other similar elements unless a sufficient portion of each neuron is present to allow its positive identification. A more common practice is to estimate neuron soma area by measuring cross sections at the plane of the nucleolus (Cook et al., '51; Matthews et al., '60; Powell and Erulkar, '62; Guillery and Stelzner, '70; Trune, '82; Peduzzi and Crossland, '83).

Measurement of neuron areas at the plane of the nucleolus permits accurate identification of neuronal populations. However, measurements of soma area obtained in this manner are affected by the position of the nucleolus with respect to the center of the cell, that is, the nucleolar eccentricity. As illustrated in Figure 1, if the nucleolus is displaced from the center of the neuron or, in experimental investigations, from its original position, this change will alter measurements of soma area. Thus it would be useful to evaluate the effect of the location of the nucleolus on measurements of soma areas and to ascertain the need for a correction of the measured value.

To evaluate the effects of nucleolar eccentricity on soma area measurements, we formulated a geometric model of the area measurement process. The model was based on a spherical soma with a plane of section through a variably located nucleolus. A set of correction factors based on the eccentricity of the nucleolus was generated. To evaluate the use of the correction factors, measurements were made on second-order auditory neurons in the chicken brain stem following cochlea removal. Removal of the cochlea from young chickens causes neuronal shrinkage and a shift in the position of the nucleolus (Born and Rubel, '85).

MODEL AND MEASUREMENTS

Geometric model

Definition. The model consisted of a sphere cut by a plane that was restricted to pass through a point. The point, representing the nucleolus, is considered to be at a given distance in a random direction from the center of the sphere. These properties assume that the soma can be represented by a sphere, that the plane of focus can be considered a plane, and that any displacement of the nucleolus from the center is not in a systematic direction. A schematic of the neuronal model is shown in Figure 2. The sphere represents the soma perimeter, the upper ellipse is the plane of section, and the point (N) is the position of the nucleolus.

Geometry. The intersection of a plane with a sphere forms a circle. If the plane passes through the center of the sphere, the area of the circle is maximum. Planes that do not pass through the center result in circular profiles that are reduced in area (Fig. 1). Determining the radius of the circle in the intersecting plane (Fig. 2) allows the area of

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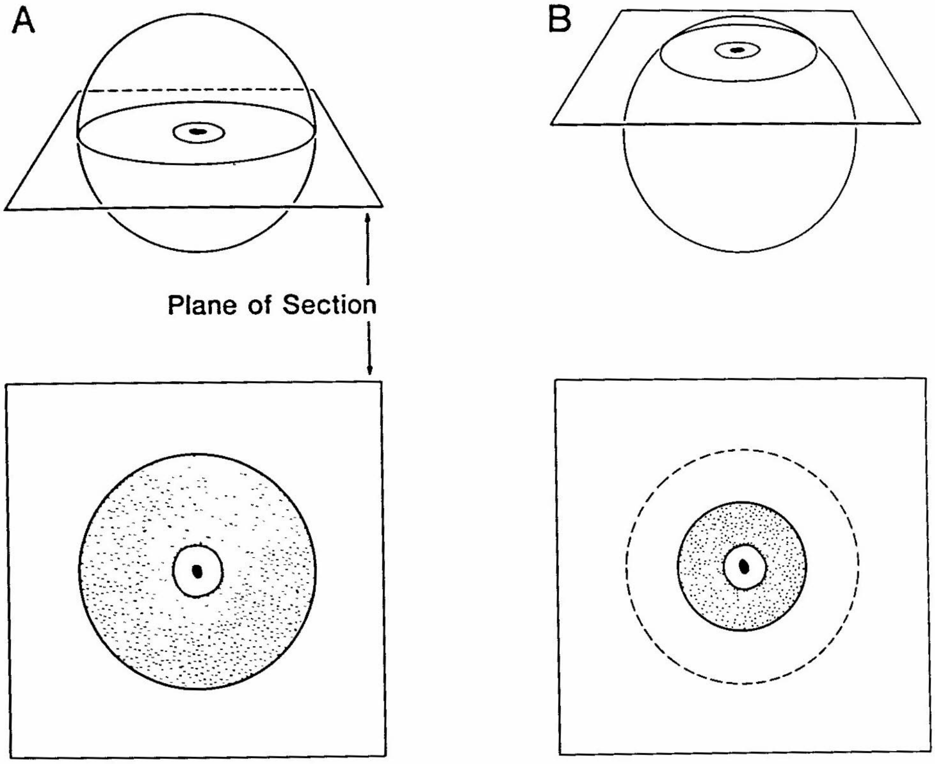

Fig. 1. Representation of how nucleolar eccentricity affects soma cross-sectional area measured at the plane of the nucleolus. In A, the nucleus and nucleolus are in the center of the soma, with a plane of section shown passing through them. When viewed in cross section, below, the observed soma cross-sectional area (stippled) has the largest possible area. If in the same neuron, the nucleus and nucleolus are displaced from the center (B) the observed cross-sectional area at the plane of the nucleolus is significantly smaller; the dashed line indicates the largest possible area.

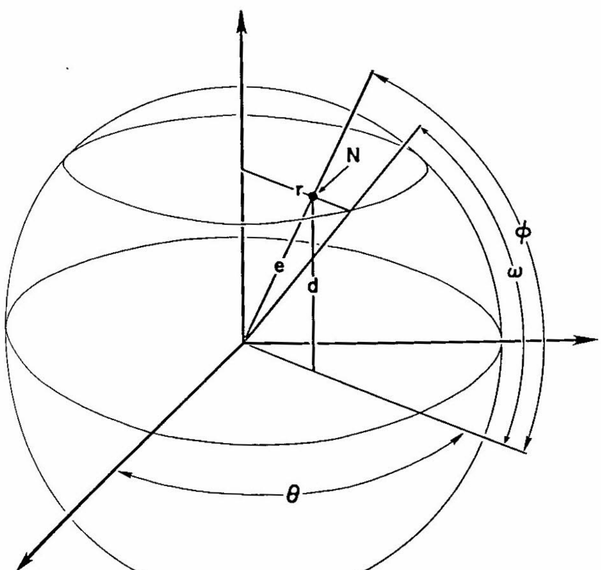

Fig. 2. Geometric model of a neuron soma cut by a plane of section through the nucleolus. The sphere represents the soma perimeter, the upper ellipse the plane of section, and the eccentric point (N) the nucleolus. The polar coordinates (see Appendix) are given for the nucleolus (e,θ,ϕ). The radius of the circle formed by the intersection of the sphere and the plane of section is r, the perpendicular distance between the center of the sphere and the circle is d, and the angle from the center of the sphere to the circle is ω.

the profile to be calculated (see Appendix). We will call the profile in the intersecting plane the observed area.

To approximate the usual paradigm for measuring neuron area, we limited the planes to those that pass through a particular point and restricted the points to a given displacement from the center, a given eccentricity. These restricted planes we will call planes of section. The mean observed area for a given eccentricity is the integral over all positions of the point with that eccentricity "weighted" for the probability of that position. This is the area expected from a cross-sectional area measurement at the plane of the nucleolus.

The integral was evaluated and a computer program was written to calculate the mean observed area for incremental positions of the nucleolus from the center of the sphere to the extreme edge. These positions were expressed in terms of percent eccentricity. The mean percent eccentricity in the plane of section (observed eccentricity) for a particular point was also calculated using the model (see Appendix). The area resulting from different amounts of eccentricity was compared to the area of the circle at the center of the sphere. A correction factor was calculated for each 1% increment in observed eccentricity (Table 1)

Neuron measurements

The cochlea was removed unilaterally from two 1-week-old chickens, and histological material was prepared as described previously (Born and Rubel, '85). Thionin-stained 10-μm coronal sections through second-order auditory neurons in n. magnocellularis (NM) were analyzed using a

Zeiss Videoplan morphometry system and a microscope with a $\times 100$ objective (N.A. 1.3). The soma cross-sectional areas of at least 30 neurons on each side of the brain were measured in two ways. The first method used the technique of focusing on the nucleolus and outlining the stained portion of the neuron. An alternative method was employed in which soma with nucleoli present in the section were outlined at the plane of the largest soma area. The percent change in soma cross-sectional area owing to deafferentation was calculated by taking the difference between the mean soma area on the side contralateral and the side ipsilateral to cochlea removal and then dividing by the mean soma area of the contralateral side.

The mean percent nucleolar eccentricity was determined by (1) measuring the distance from the nucleolus to the plasma membrane along the line that passes through the nucleolus and the center of the soma; (2) measuring the soma diameter along the same line; and (3) calculating the observed nucleolar eccentricity by subtracting the distance from the nucleolus to the plasma membrane from one-half of the diameter (the radius); (4) expressing the eccentricity as a percent by dividing this value by the radius and multiplying by 100. If the cell orientations are random with respect to the plane of section, the mean eccentricity determined in this manner is the same as the observed eccentricity calculated by the geometric model. For this study we determined the eccentricity in the same neurons for which the area was measured.

To verify that the properties of NM neurons were consistent with the model, two additional features were analyzed. An estimate of the circularity of the soma was made by taking the ratio of the shortest diameter to the longest diameter. A ratio of 1 indicates a perfect circle, while ratios less than one indicate deviations from circularity. To determine if any displacement of the nucleolus was polarized with respect to the plane of section, horizontal sections were prepared from an experimental animal. The mean percent eccentricity was determined as for the coronal sections. If displacement of the nucleolus does not occur in any consistent direction, the two measurements will be equal.

RESULTS

Geometric model

The mathematical model produced soma areas that were reduced in size depending on the degree of nucleolar eccentricity. Figure 3 shows the difference between mean observed area and the cross-sectional area of the circle at the center of the sphere expressed as a percent of the cross-sectional area at the center of the sphere. Since the observed area decreases with increasing eccentricity the error increases, although not linearly. The amount of reduction in observed cross-sectional area for various eccentricities was used to calculate correction factors (see Table 1).

Measurements

Average ($\pm$ S.D.) soma cross-sectional area in the plane of the nucleolus for n. magnocellularis neurons contralateral to cochlea removal was 367.3 $\pm$ 48.7 μm^2. On the side of the brain ipsilateral to the cochlea removal, the mean soma cross-sectional area at the plane of the nucleolus was 298.9 $\pm$ 54.2 μm^2. This is a reduction of 18.6% for the side ipsilateral to the cochlea removal. These measurements are comparable with previous measurements of soma area at this level in NM. The difference between the two sides of the brain is attributed to shrinkage of the ipsilateral neurons, since the contralateral NM neuron area has been shown to be unaffected by cochlea removal (Born and Rubel, '85).

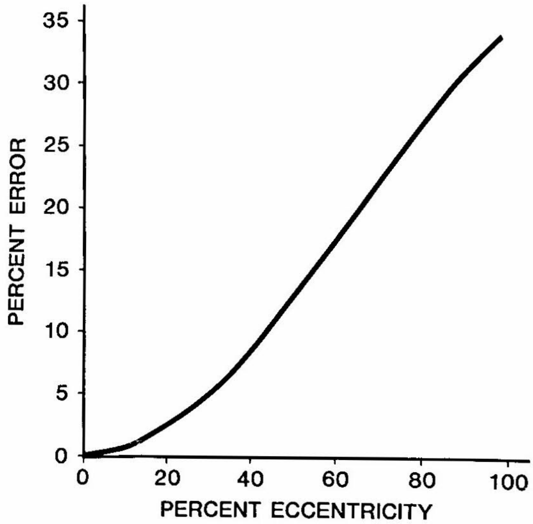

Fig. 3. Percent error of mean "observed" area as a function of percent nucleolar eccentricity. The percent error is the difference between the mean observed area and the cross-sectional area of the circle at the center of the sphere expressed as a percent of the cross-sectional area at the center of the sphere. The line shows that such a measurement represents an increasing error relative to the true area.

The position of the nucleolus was visibly different in neurons on the two sides of the brain. Figure 4 shows photomicrographs of representative neurons. On the side contralateral to cochlea removal the nucleolus appeared generally near the center of the neuron, while on the side ipsilateral to cochlea removal the nucleolus was often displaced from the center. The average position of the nucleolus on the unoperated side of the brain was 18.4% eccentric. On the operated side of the brain the mean eccentricity was 34.9%. While there was considerable variability in the degree of eccentricity on the experimental side, the mean eccentricity was significantly greater than the mean on the control side ($t = 5.99$, $P < .01$).

From Table 1 the correction factors for these percent eccentricities are 1.019 and 1.067 for the sides contralateral and ipsilateral to cochlea removal, respectively. Applying these correction factors, the average neuron area for the control side is 374.3 μm^2 and for the experimental side is 318.9 μm^2, a difference of 14.8%.

Neurons with a nucleolus measured at the plane of the largest cross-sectional area had uncorrected mean neuron areas of 368.0 μm^2 and 305.1 μm^2 for the control and experimental sides, respectively. These values differ by 16.9%. Compared to the measurements at the plane of the nucleolus the mean for the experimental side is altered more than for the control side. However, neither of these values agrees precisely with the corrected areas.

The somata of n. magnocellularis neurons appear generally circular in cross section (Fig. 4). The form factor for NM ipsilateral to cochlea removal was 0.89 and contralateral was 0.90. The difference is small and was not statistically significant ($t = 0.3$). In horizontal sections the nucleolar eccentricity of NM neurons was 20.7% on the contralateral side and 35.6% on the side ipsilateral to cochlea removal. These eccentricities are not significantly different from those found in coronal sections.

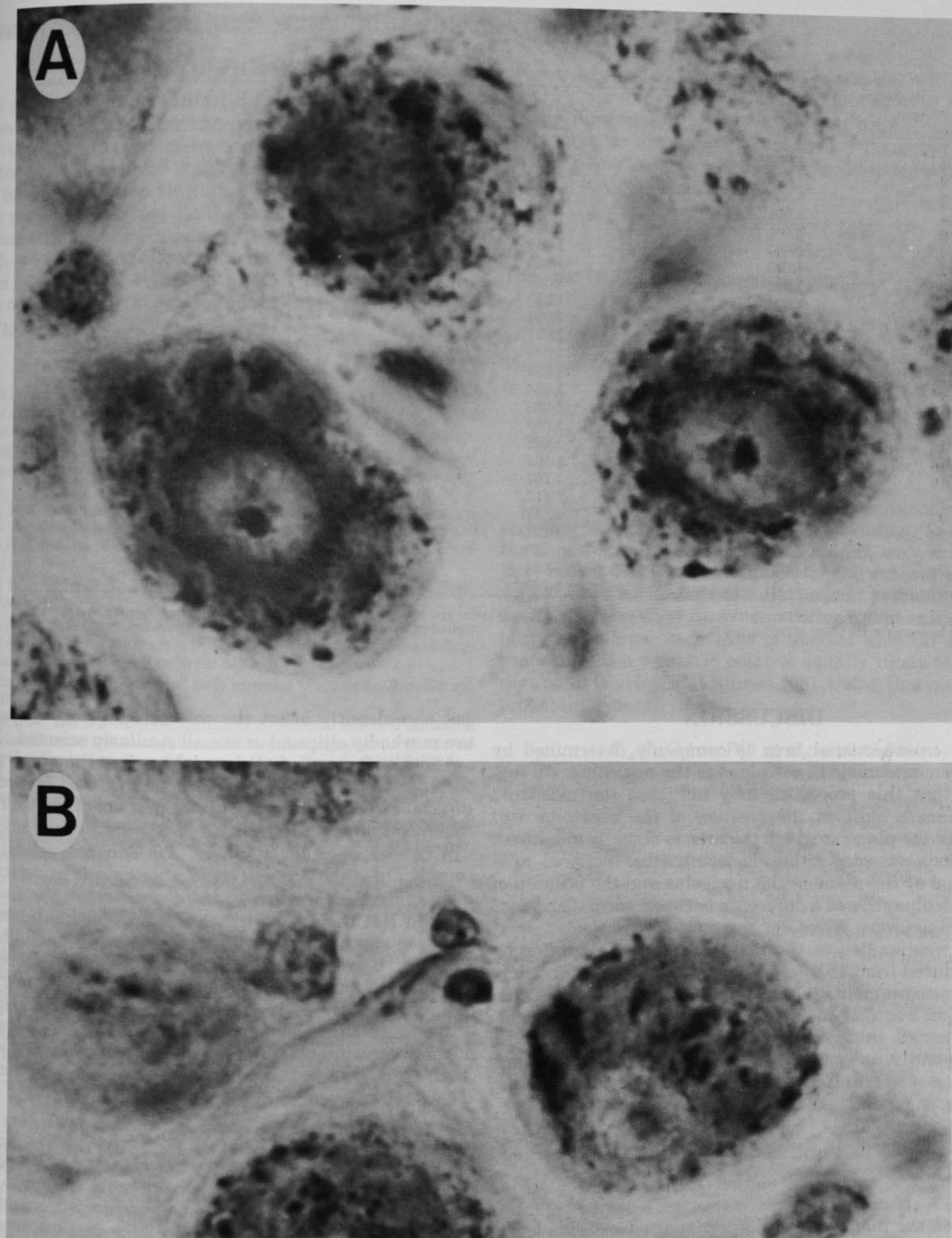

Fig. 4. Photomicrographs from nucleus magnocellularis neurons on the side contralateral (A) and ipsilateral (B) to cochlea removal. The cochlea was removed from a 6-week-old chicken, and they were sacrificed 26 days later. The somata are roughly circular in cross section, but the nucleolus on the deafferented side (B) is displaced from the center compared to the contralateral normal side (A). Thionin-stained 10-μm coronal section.

TABLE 1. Area Measurement Correction Factors for Different Percent Nucleolar Eccentricity[1]

Percent eccentricity	Correction factor	Percent eccentricity	Correction factor	Percent eccentricity	Correction factor	Percent eccentricity	Correction factor
1	1.000	26	1.037	51	1.144	76	1.321
2	1.000	27	1.040	52	1.150	77	1.329
3	1.000	28	1.043	53	1.156	78	1.337
4	1.001	29	1.046	54	1.162	79	1.345
5	1.001	30	1.049	55	1.168	80	1.353
6	1.002	31	1.053	56	1.175	81	1.361
7	1.003	32	1.056	57	1.181	82	1.370
8	1.003	33	1.060	58	1.187	83	1.378
9	1.004	34	1.063	59	1.194	84	1.386
10	1.005	35	1.067	60	1.201	85	1.394
11	1.007	36	1.071	61	1.207	86	1.402
12	1.008	37	1.075	62	1.214	87	1.410
13	1.009	38	1.079	63	1.221	88	1.418
14	1.011	39	1.084	64	1.228	89	1.426
15	1.012	40	1.088	65	1.236	90	1.434
16	1.014	41	1.093	66	1.243	91	1.442
17	1.016	42	1.097	67	1.250	92	1.450
18	1.018	43	1.102	68	1.258	93	1.457
19	1.020	44	1.107	69	1.265	94	1.464
20	1.022	45	1.112	70	1.273	95	1.471
21	1.024	46	1.117	71	1.281	96	1.478
22	1.026	47	1.122	72	1.289	97	1.484
23	1.029	48	1.128	73	1.296	98	1.490
24	1.031	49	1.133	74	1.304	99	1.496
25	1.034	50	1.139	75	1.312	100	1.500

[1]To apply the correction find the average percent eccentricity of the nucleolus and multiply the associated correction factor times the average area measured at the plane of the nucleolus.

DISCUSSION

Soma cross-sectional area is commonly determined by measurements made at the plane of the nucleolus. We suggested that this procedure may influence the measured area, since a shift in the position of the nucleolus will influence the plane at which the area is actually measured. In an experimental situation, soma cross-sectional area measured at the plane of the nucleolus and the position of the nucleolus showed a difference between normal and deafferented neurons. When our correction for nucleolar eccentricity was applied to the measured area, this difference was reduced from 18.6% to 14.8%. An alternate procedure, using measurements taken at the largest observable cross section in neurons cut through the nucleolus, approached the corrected value but still overestimated the difference. Consequently, measuring at the largest observable cross section is not a suitable method for estimating cross-sectional area for sections of this thickness.

Suitability of the model

A geometric model was chosen to reflect the measuring process. A sphere cut by a plane through a point adequately describes the method. The properties used in formulating the model are consistent with findings in n. magnocellularis and probably apply to many parts of the brain. One property of the model is spherical shape. As indicated by the ratio of longest to shortest diameter, NM neurons are roughly spherical. As soma form markedly deviates from spherical the model may become unsuitable. Although we have not yet solved the geometry for other shapes, the deviations found in many neuron somata should have minimal effect on the correction factors. Dendritic protusions from the soma do not affect the application of the correction factors unless they account for a large portion of the soma area in the plane of section. Elliptical somata also should not significantly affect the correction factors unless they are markedly ellipsoid or are all similarly oriented.

Another feature of the model is that the plane passes through a point that is a given distance *and in a random direction* from the center. This property of random direction assumes that the distribution of positions of the nucleolus can be described as a sphere. We can not validate this completely, although systematic biases can be detected by examining the eccentricity in perpendicular planes of section. In NM the eccentricities found in horizontal sections were not significantly different from those found in coronal sections. If there is a statistical difference between the eccentricity found in the two planes, the magnitude of the difference becomes important. For small differences the correction factors should probably be used in whatever plane is more convenient. If the eccentricity in one plane is more than twice that in the other, then the correction factors in Table 1 are no longer appropriate.

Applications

Modeling the area measuring scheme commonly used indicated that there can be considerable influence of the position of the nucleolus on soma area measurements. The effect is graded from no effect to a 33% reduction, depending on the degree of nucleolar eccentricity. The magnitude of the resulting correction factor increases with increasing mean eccentricity although it is not a linear relationship.

The correction factors can be applied to neurons that reasonably fit the assumptions outlined above. To determine the appropriate correction (1) measure the soma area of a sample of neurons at the plane of the nucleolus; (2) measure the position of the nucleolus in a subsample and calculate its mean eccentricity as described in the Model and Measurements section; (3) look up the correction factor in Table 1 for this eccentricity; and (4) multiply the measured area by the correction factor.

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APPENDIX

The model consists of the following parts: a sphere with unit radius centered in a Cartesian coordinate system, an eccentric point within the sphere, and a plane parallel to the x-y plane of the coordinate system, which contains the point (see Fig. 2). The intersection of the plane with the sphere forms a circle, with the point in the interior of the circle. To model the measurement process we want to find the expected value for the area of the circle (a weighted average over all positions of the point at a given distance from the center of the circle). To use the model with measures of eccentricity made on sections we also need the expected value for the eccentricty of the point in the circle.

The eccentric point can be represented in spherical coordinates by $E(e,\theta,\phi)$; e is a dimensionless constant, with range $0 \leqslant e \leqslant 1$, formed by dividing the distance of the point from the center of the sphere by the radius of the sphere; and θ,ϕ are random variables with ranges $0 \leqslant \theta \leqslant 2\pi$ and $-\pi/2 \leqslant \phi \leqslant \pi/2$. The radius of the circle in the intersecting plane (r) is given by $r = \cos(\omega)$. The perpendicular distance (d) from the x-y plane to the intersecting plane is $d = e \cdot \sin(\phi)$. From geometric relationships $e \cdot \sin(\phi) = \sin(\omega)$, thus

$$r = \cos(\omega) = \sqrt{[1 - e^2 \cdot \sin^2(\phi)]}.$$

The area (A_c) in the intersecting plane is

$$A_c(e,\theta,\phi) = \pi r^2 = \pi[1 - e^2 \cdot \sin^2(\phi)].$$

The eccentricity (E_c) of the point in the intersecting plane is

$$E_c(e,\theta,\phi) = \frac{d}{r} = \frac{e \cdot \cos(\phi)}{\sqrt{[1 - e^2 \cdot \sin^2(\phi)]}}.$$

The position of the eccentric point was modeled by restricting it to lie on a sphere of fixed radius. To evaluate the expected value functions for A_c and E_c we will need the joint probability density function for the random variables $f_{\theta,\phi}(\theta,\phi)$. This can be derived from the joint probability distribution function $F_{\theta,\phi}(\theta,\phi)$. We can define a measure of probability as follows: each unit of surface area on a sphere containing the eccentric point is equally likely to contain that point (DeHoff and Rhines, '68). Using this measure of probability we can find

$$F_{\theta,\phi}(C_\theta,C_\phi) = P\{\theta \leqslant C_\theta;\ \phi \leqslant C_\phi\} = \frac{\int_{-\pi/2}^{C_\phi}\int_0^{C_\theta} \Delta SA\, d\theta d\phi}{\int_{-\pi/2}^{\pi/2}\int_0^{2\pi} \Delta SA\, d\theta d\phi},$$

where C_θ and C_ϕ are constants and ΔSA is given by $e^2\cos(\phi)$. Thus,

$$F_{\theta,\phi}(\theta,\phi) = \frac{\phi[1 + \sin(\phi)]}{4\pi} \text{ and}$$

$$f_{\theta,\phi}(\theta,\phi) = \frac{\partial^2 F_{\theta\phi}(\theta\phi)}{\partial\theta\,\partial\phi} = \frac{1}{4\pi}\cos(\phi).$$

The expected values can be found by using the calculus of probability where $E\{x\} = \int x f_x(x)\,dx$. The expected value for the area of the circle is

$$E\{A_c(e,\theta,\phi)\} = \int_{-\pi/2}^{\pi/2}\int_0^{2\pi} \pi[1 - e^2\sin^2(\phi)]\, f_{\theta,\phi}(\theta,\phi)\, d\theta d\phi = \frac{\pi}{3}(3 - e^2).$$

The expected value for the eccentricity is

$$E\{E_c(e,\theta,\phi)\} = \int_{-\pi/2}^{\pi/2}\int_0^{2\pi} \frac{e \cdot \cos(\phi)}{\sqrt{(1 - e^2\sin^2(\phi))}}\, f_{\theta\phi}(\theta,\phi)\, d\theta d\phi.$$

This integral can be approximated to an arbitrary accuracy using two special functions called elliptic functions. The equations are

$$= \left(\frac{1}{e}\right)[E - (1 - e^2)K]$$

$$E = \frac{\pi}{2(1+m)}\left[1 + \left(\frac{1^2}{2^2}\right)m^2 + \left(\frac{1^2}{2^2 \cdot 4^2}\right)m^4 + \left(\frac{1^2 \cdot 3^2}{2^2 \cdot 4^2 \cdot 6^2}\right)m^6 + \cdots\right]$$

$$K = \frac{\pi(1+m)}{2}\left[1 + \left(\frac{1^2}{2^2}\right)m^2 + \left(\frac{1^2 \cdot 3^2}{2^2 \cdot 4^2}\right)m^4 + \left(\frac{1^2 \cdot 3^2 \cdot 5^2}{2^2 \cdot 4^2 \cdot 6^2}\right)m^6 + \cdots\right]$$

$$m = \frac{1 - \sqrt{(1 - e^2)}}{1 + \sqrt{(1 - e^2}}.$$

The elliptic functions were evaluated to six significant figures using Fortran IV on a DEC PDP 11/23. Note that both of the expected value equations are functions of only the distance (e) from the point to the center of the sphere.

The results are expressed in relation to the area of a circle at the center of the sphere (A_a) to remove any dependence on the actual size of the sphere. The real area (A_a) is given by πR^2, where R is the radius of the sphere. The percent error was calculated for each 1% increment in E_c as $(A_a - A_c)/A_a \cdot 100$, and is plotted in Figure 3. The correction factor is A_a/A_c. The table of correction factors and the error plot were generated by calculating the eccentricity in the sphere given the expected eccentricity in the table.