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THE IMPORTANCE OF SECTION THICKNESS AND PARTICLE DIAMETER RATIO IN NUMBER PER UNIT VOLUME ESTIMATIONS EVALUATED BY COMPUTER SIMULATION (PRELIMINARY REPORT)

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ABSTRACT

Computer simulation has been carried out on a model consisting of spheres of diameter D = 7 µm, randomly distributed in a cube with edge length 300 µm. The cube was sistematically cut into slices of thickness t (0 µm ≤ t ≤ 20 µm). Four categories of stereological methods for estimating the number per unit volume for spheres were used : methods for true sections or for relatively thin sections (Wicksell, Weibel-Gomez, DeHoff, DeHoff's method modified by Weibel), methods for relatively thick sections (Abercrombie, Floderus), method for differential counting of particles (Ebbeson-Tang, Ebbeson-Tang's modified by Kališnik and Pajer) and methods for counting particles in three dimensional space (Sterio, Sterio's method modified by Howard et al.). For all methods under investigation the range of the ratio t/D where the relative deviation of the number per unit volume falls within acceptable limits of ± 10 % was established. The results show that three out of four methods for true or relatively thin sections lead to acceptable results under the condition t/D ≤ 1/7. Among the methods for relatively thick sections the results approach acceptable values at ratios t/D > 2.5. Among the methods for differential counting of particles results are acceptable at ratios t_1/D > 2.5, for the original method of Ebbeson-Tang. Ebbeson-Tang's modification by Kališnik and Pajer leads to acceptable results in all cases under investigation. The use of a disector in the original Sterio method highly underestimates the number per unit volume. Only the modification of Sterio's method by Howard et al. leads to acceptable results at the ratio t/D > 3. It will be necessary to test the validity of the above findings on a model with higher resolution.

KEYWORDS: computer simulation, numerical density, resolution, section thickness, sphere diameter

INTRODUCTION

There are several stereological methods for determining the mean number of particles per unit volume, N_V. These methods can be divided into four main categories : methods for true sections or for relatively thin sections (Wicksell, 1925, 1926; Weibel-Gomez, 1962; DeHoff, 1964; DeHoff's method modified by Weibel, 1979, 1980), methods for relatively thick sections (Abercrombie, 1946; Floderus, 1944), methods for differential counting of particles (Ebbeson-Tang, 1965; Ebbeson-Tang's method modified by Kališnik and Pajer, 1985) and methods for counting particles in three dimensional space (Sterio, 1983; Sterio's method modified by Howard et al., 1985).

With the methods for relatively thin sections, the use of very thin slices or true sections has been proposed. Very thick slices are recommended when applying the methods for relatively thick sections. Methods for differential counting require the use of as differently thick slices as possible. Double counting of the same particle is prevented by using the principle of forbidden lines in all previous cases. The principle of forbidden planes is employed for the methods for counting particles in three dimensional space.

The allowed limits of the ratio of the slice thickness t and the particle diameter D, t/D, has not been given for any of these methods so far. The aim of the present study was to establish the range of the ratio t/D where the relative deviation of N_V for an individual method falls within acceptable limits ± 10 %.

We decided to solve this problem by using computer simulation. The reason for this is that empirical testing of an individual method by cutting an object and counting the particles would not be feasible practically due to the numerous combinations of the variables. Furthermore, because one and the same physical object could not be cut repeatedly, the results obtained would not have the same validity as the results obtained by computer simulation.

Several authors have used a computer simulation in solving stereological problems. Among others Durand and Warren (1981) have used simulation for evaluating empirical formulae for N_V in systems of arbitrarily shaped particles in opaque sections. We have not found any literature data regarding computer simulation that examined the effect of the section thickness and particle diameter ratio on the applicability of individual stereological methods for the evaluation of N_V.

METHODS

Computer simulation

The program is written in Pascal for the Partner Iskra Delta personal computer, with operating system CP/M 3.0 (Control Program for Minicomputers, version 3.0) and 128 Kb of user RAM (Random Access Memory). External memory : hard disk 10 Mb, floppy disk 800 Kb.

The program consists of two parts :

- generation of the particle configuration (Fig. 1) and
- simulation of stereological methods for counting particles (Fig. 2).

The model for analysis is a cube with edge length L = 300 µm. The spheres with diameter D = 7 µm are randomly distributed in the cube, their number being $N = 13 \times 10^3$. The particles do not overlap, the smallest distance permitted between the centers of the two particles is 8 µm. The number per unit volume is $N_V = 4.815 \times 10^5/mm^3$. The volume fraction of ideal spheres with the diameter D would be 0.0865. After the initialization of the parameters N, L and D, the cartesian coordinates of the centers of the particles (x_i, y_i, z_i) are generated considering the above restrictions. After the generation of the particles is completed their coordinates are stored in the file.

The second part of the program is used for the analysis of the generated model. The cube is cut sistematically into slices of thickness t (0 µm ≤ t ≤ 20 µm). The analysis consists of four parts :

- analysis for true or relatively thin sections
- analysis for relatively thick sections
- analysis for differential counting of particles
- analysis for counting particles in three dimensional space

For all the methods the counting is simulated on the test area $A_t = 9.31 \times 10^3$ µm², with the test grid M42, the distance between two neighbouring test points being d = 16 µm. Sampling was systematic and complete in one section. For analysis of relatively thin sections the numbers per unit volume are determined at section thicknesses 0 µm ≤ t ≤ 4 µm, and in all the other methods at 2 µm ≤ t ≤ 20 µm.

Statistical analysis

The relative deviations of the number of particles per unit volume from the true value ($\Delta N_V/N_V$) for individual methods were determined. For a sample with m sections the relative standard error was estimated as

$$RSE = \left\{ \sum_{i=1}^{m} (N_{Vi} - \bar{N}_V)^2 / \left[(m-1) m \bar{N}_V^2 \right] * (M-m)/M \right\}^{1/2} ,$$

where 15 ≤ M ≤ 300 is the maximal number of slices. The range of the parameter t/D where the relative deviation $\Delta N_V/N_V$ falls within acceptable limits of ± 10 % was estimated for each of the methods considered. The resolution (one pixel, the distance between two neighbouring points on the same coordinate) was 1 µm.

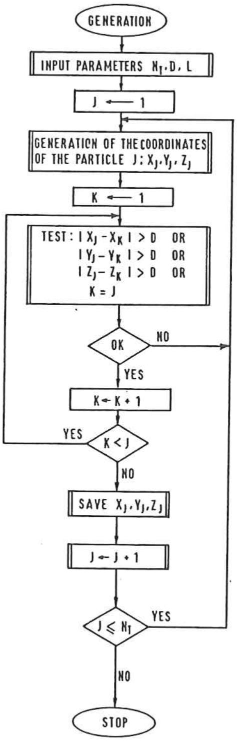

Fig. 1 : Flow-chart for generation of particles in three dimensional space

N_T - total number of particles
D - diameter of particles
L - length of the side of the cube
J - number of generated particles
K - index of already generated particles

Coordinates (x_J, y_J, z_J) are in interval (1,300)

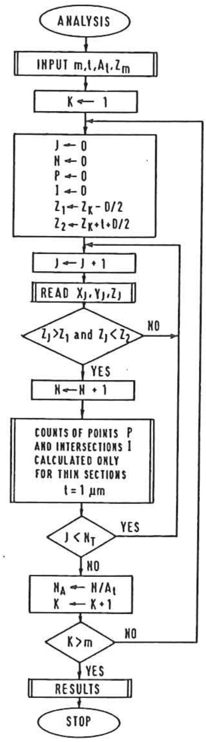

Fig. 2 : Flow-chart for stereological analysis of the computer model

m - number of slices
t - section thickness
A_t - test area
Z_m - the position of the slice m in the model
K - index for slices
J - index for particles
N - counter for particle profiles
P - counter for particle hits
I - counter for intersections
D - diameter of the particle
RESULTS - subroutine determining N_V and RSE
N_T - total number of particles

RESULTS

The results of counting particles in 15 sections are presented graphically (Figs. 3-6). For all the data presented the relative standard error was below 5 %.

In comparing methods for true or relatively thin sections the results for three out of four methods lie in the interval ± 10 % N_V at the infinitesimal section thickness $t \rightarrow 0$. These are : Wicksell, Weibel-Gomez and DeHoff. DeHoff's method modified by Weibel gives a relative deviation $\Delta N_V/N_V$ greater than - 20 %. With increasing section thickness the relative deviations increase linearly. At the ratio t/D = 1/7 the results for all the methods but DeHoff's are acceptable (Fig. 3).

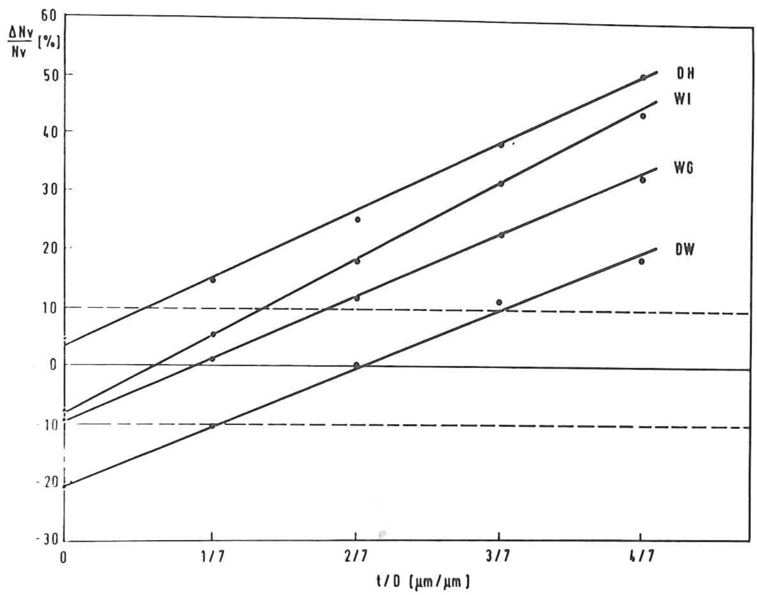

Fig. 3 : Relative deviation of the number per unit volume ($\Delta N_V/N_V$) for methods for true or relatively thin sections (DH - DeHoff, WI - Wicksell, WG - Weibel-Gomez, DW - DeHoff-Weibel) as a function of the section thickness (t) and particle diameter (D) ratio, t/D.

Of the methods for relatively thick sections the Floderus method gives lower relative deviations in N_V than Abercrombie's method. For both methods, the relative deviation of N_V becomes lower with increasing section thickness. But even so, at the ratio t/D > 2.5, the results obtained by the Floderus method are underestimated by -10 %. At t = 20 μm the error is even bigger (Fig. 4).

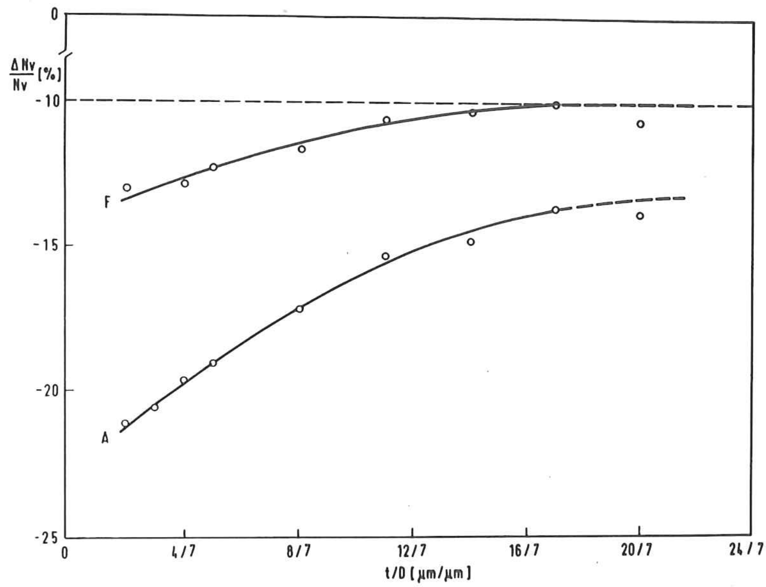

Fig. 4 : Relative deviation of the number per unit volume ($\Delta N_V/N_V$) for methods for relatively thick sections (A - Abercrombie, F - Floderus) as a function of the section thickness (t) and particle diameter (D) ratio, t/D.

Methods of differential counting of particles give lower relative deviations of N_V for bigger differences in the thickness of the two sections: thicker section t_1 and thinner section t_2. For the ratio $t_1/D > 2.5$ both methods give results within the acceptable interval ± 10 % N_V. The modified Ebbeson-Tang method, where the particles are firstly counted through all the section thickness and secondly in the same section equal to the depth of focus, gives acceptable results for all the ratios t/D under investigation (Fig. 5).

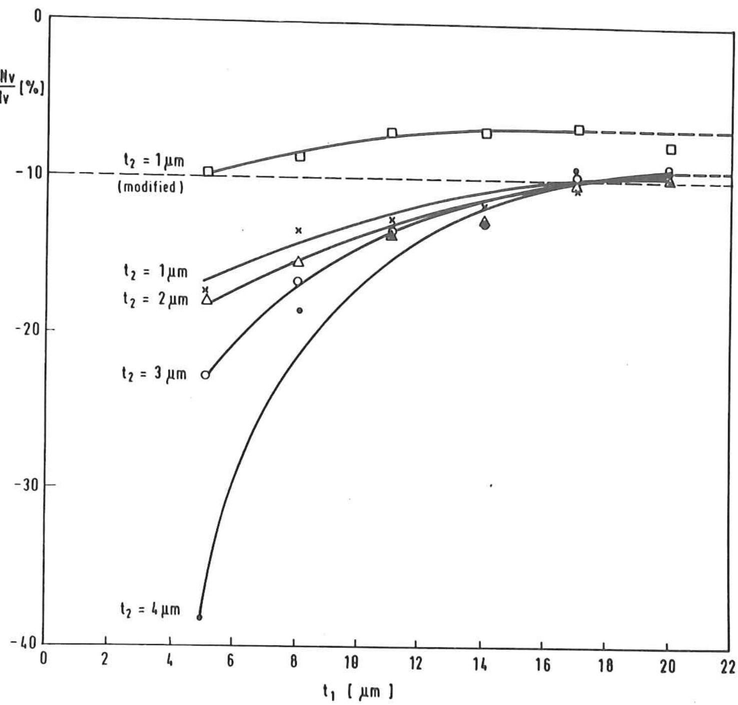

Fig. 5 : Relative deviation of the number per unit volume ($\Delta N_V/N_V$) for methods of differential counting of particles of diameter D = 7 µm (Ebbeson-Tang and modification by Kalisnik and Pajer) as a function of different section thicknesses of the thicker slice (t_1) and thinner slice (t_2).

The disector method for counting particles in three dimensional space gives highly underestimated results for relatively thin sections (Fig. 6, left half). With increasing thickness of the section the relative deviation of N_V decreases, and the results become acceptable at the ratio t/D > 3 (Fig. 6, right half).

From the results presented above we can conclude that all the methods but those from the category of true or relatively thin sections, underestimate the values of the number per unit volume. Underestimation increases with decreasing thickness of the section. In the category of methods for thin sections at t = 0 µm all the methods but DeHoff's underestimate the values of number per unit volume. With increasing thickness of the section, the results for these methods become overestimated.

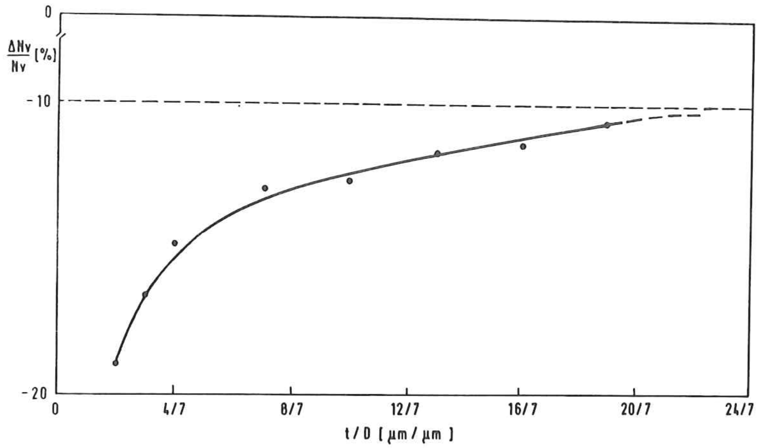

Fig. 6 : Relative deviation of the number per unit volume ($\Delta N_V/N_V$) for methods of counting particles in three dimensional space (Sterio, left half of the curve, modification by Howard et al., right half of the curve) as a function of the section thickness (t) and particle diameter (D) ratio, t/D.

DISCUSSION

A possible deficiency of the computer simulation carried out in this work can be the consequence of the low resolution of the system under investigation. One pixel had the value of 1/7 of the particle diameter. It has been estimated that the volume of the particles encountered in the simulation was approximately 4 % lower than expected for spheres of this diameter. This effect would contribute to the underestimated numbers per unit volume for practically all the methods under investigation. It would be necessary to test the validity of the above results on a model with essentially higher resolution. This can easily be done by increasing the number of pixels from which the particle is composed. One can expect that with higher resolution the volume of the particles encountered in the simulation would approach the volume of ideal spheres of the same diameter.

Another weakness of the model becomes visible when analysing the methods for relatively thick sections and for methods for differential counting of particles. Relative deviations in N_V become larger for the section thickness t = 20 µm, compared to lower thicknesses. At this section thickness the model was completely "exhausted", the consequence being the lack of the referential space for·2(R-h), where h is the height of the lost cap, and R = D/2.

In the category of methods for true and relatively thin sections we found that the results for individual methods are different, in spite of the fact that all the equations have the same theoretical background. DeHoff's method modified by Weibel consists of a transformation of the equation that is mathematically correct. Different results obtained by computer simulation are probably due to the different number of approximately determined parameters (i.e. volume and surface density of particles in DeHoff's method modified by Weibel, or only surface density in DeHoff's method or volume density in the Weibel-Gomez method) leading to different magnitudes of errors.

The results of methods in the category for relatively thick sections are in agreement with expectations; the reason for underestimated results has been discussed previously.

Comparing methods for differential counting of particles, the modified method where the second counting is performed in the same section at a depth of focus equal to 1 µm instead of counting the particles in the thinner section t_2=1 µm gives better results. This is a further advantage of the modified method compared to the original Ebbeson-Tang method, in addition to the practical one of not requiring two series of differently thick slices.

The results from the methods for counting particles in three dimensional space are at first sight surprising. An apparently theoretically well established method, which is supposed to reproduce unbiased results, turned out to be inapplicable for relatively thin sections. As has been originally proposed (Sterio, 1983), the sections should be thinner than the height of the particle perpendicular to the plane of cutting - in our model that means that the section thickness shoud be thinner than the diameter of the particle D = 7 µm. We have no explanation for the underestimated numbers per unit volume obtained by the Sterio method so far. Sterio's method modified by Howard et al. (1985) and the use of a Tandem Scanning Reflected Light Microscope (TSRLM) give useful results under the condition that the section thickness is relatively big, $t > 3D$! In the case of $t = 20$ µm we are dealing not with sampling but with an inventory of the particles in the reference space.

In spite of some deficiences in the approach described, the results of this study show the usefulness of computer simulation for evaluating the applicability of different methods for estimating the particle number per unit volume. The importance of the proper choice of the ratio of the section thickness t, and particle diameter D, for an individual method has been demonstrated and optimal ranges of t/D for different methods have been estimated.

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