AUTOMATED RADIOAUTOGRAPHIC GRAIN COUNTING: CORRECTION FOR GRAIN OVERLAP

W.H. Schuette*, S.S. Chen, S.J. Occhipinti,
H.S. Mujagic, and S.E. Shackney

*Applied Clinical Engineering Section,
Biomedical Engineering & Instrumentation Branch,
Division of Research Services
and
Cellular Kinetics Section,
Clinical Pharmacology Branch,
Division of Cancer Treatment,
National Cancer Institute

National Institutes of Health
Bethesda, Maryland 20205

*To whom reprint requests should be sent.

ABSTRACT

An algorithm is described for the calculation of radioautographic cell grain count from measurements of total cell nuclear area and total grain area. This algorithm provides a statistical correction for grain overlap that is based on the solution to the occupancy problem in probability theory. This method permits the use of automated grain counting over a wide range of grain counts/cell, and extends the useful dynamic range of radioautographic grain counting to well over 200 grains/cell.

Several experimental studies have suggested that radioautographic labeling intensity following pulse exposure to tritiated thymidine (^{3}HTdR) can reflect the rates of DNA synthesis in individual cells (Terasima & Tolmach, 1963; Dendy & Cleaver, 1964; Dendy & Smith, 1964; Hale & Cooper, 1965; Wright, 1971; Sordat et al, 1972, Tolmach, Weiss & Hopwood, 1972; Shackney, Ford & Wittig, 1973; Shackney & Ford, 1974; Dormer et al, 1975; Hirt & Wagner, 1975). Under proper conditions, radioautographic labeling intensity can also be used to monitor rapid changes in the rate of DNA synthesis in individual cells following exposure to cytotoxic drugs (Ford & Shackney, 1977; Shackney et al, 1980). Although such information could be of considerable value in a variety of cell kinetic studies, few investigators are prepared to undertake such studies, owing largely to the tedious and time-consuming nature of radioautographic grain counting.

Automated image analysis systems can simplify the task of radioautographic grain counting considerably. Several years ago, we reported on the practical utility of a television-based automated grain counter (Bunn, Ford & Shackney, 1977). In that study a major unresolved problem was the failure of the image analysis system to resolve and count separately grains that overlapped one another, necessitating a visual correction of the instrument count. This visual grain count correction could be a tedious and time-consuming process in itself, especially in heavily labeled cells where grain overlap may be extensive.

Alternatively, it is possible to obtain an estimate of grain count by measuring the total area covered by the grains and dividing the total grain area by the area subtended by a single grain. Extensive grain overlap can still lead to an underestimation of the true grain count, especially in heavily labeled cells with small nucleii (Kraemer, Buchanan & Stinson, 1980). However, the problem of overlapping grain areas is far more tractable than that of distinguishing overlapping grains as separate objects for enumeration.

In this paper, we describe an algorithm for determining nuclear grain counts from measurements of radioautographic grain area and cell nuclear area. This algorithm provides a statistical correction for grain overlap that is based on the solution to a more general problem in probability theory known as the occupancy problem.

MATERIALS AND METHODS

The Occupancy Problem and its Application to Grain Counting

When n balls are distributed randomly among m boxes, and when there is no restriction on the number of balls that any one box can contain, the expected number of empty boxes remaining is given by (Dwass, 1970):

$$\frac{(m-1)^{n}}{m^{(n-1)}} \qquad [1]$$

In applying this equation to the grain counting problem, the balls correspond to grains, and the boxes correspond to

cell nuclear areas, expressed in units of average area per individual grain. That is,

$$n = \text{true grain count} \qquad [2]$$

and,

$$m = \frac{\text{total nuclear area}}{\text{area of an average sized grain}} \qquad [3]$$

The number of occupied boxes corresponds to the observed grain area, and is given by,

$$m - \frac{(m-1)^n}{m^{(n-1)}} \qquad [4]$$

The ratio of observed grain area to total nuclear area, R, is given by,

$$R = 1 - \frac{(m-1)^n}{m^n} \qquad [5]$$

Solving for n, the true grain count,

$$\text{true grain count} = \log (1-R)/(\log(m-1)-\log m) \qquad [6]$$

Experimental Procedures

Feulgen-stained Cytospin (Shandon Instruments, Sewickly, Pennsylvania) slides were prepared of human lymphoma cells that had been exposed to ^{3}HTdR *in vitro* at 37°C for one hour at a final concentration of 2 Curie/ml. Radioautographs were prepared using NTB2 liquid emulsion, as described previously (Shackney, Ford & Wittig, 1973).

Total grain area per cell and cell nuclear area were measured by means of an Artek model 800 image analyzer

(Artek Corp., Farmingdale, NY). Nuclear boundaries were detected by means of an edge detection circuit. Nuclear boundaries could be corrected by the operator by means of a light pen. Grain area boundaries were determined by combining edge detection with an operator-controlled threshold setting.

Grain area per cell and cell nuclear area were measured in 1000 cells. Visual grain counts were recorded for the same cells. The area of an average-sized grain was determined separately from measurements of grain area and visual grain counts in 50 lightly labeled cells (<10 grains/cell) that had no detectable grain overlap by visual inspection.

Cell grain counts were calculated in two ways. The total grain area divided by the value for the area per average-sized grain was taken as a cell grain count that was <u>not</u> corrected for grain overlap. The grain count obtained from equation 6, above, was taken as the grain count that was corrected for grain overlap.

RESULTS AND DISCUSSION

A cell-by-cell comparison of grain counts obtained from grain area measurements without correction for grain overlap and visual grain counts is shown in Figure 1. When visual grain counts exceed 50-75 grains/cell, the overwhelming majority of data points falls below the 45 degree line of

identity. Total grain area/cell does not increase linearly with increasing grain count because of extensive grain overlap in cells with high grain counts.

A comparison of grain counts obtained from grain area measurements with correction for grain overlap (equation 6) and the visual grain counts is shown in Figure 2. It is apparent that the calculated grain counts correspond with the visual counts over the entire grain count range studied.

Thus, the correction for grain overlap permits the use of automated grain counting over a wide range of grain counts, and even extends the useful dynamic range for radio-autographic grain counting to well over 200 grains/cell (provided, of course, that cytomorphologic details that might be obscured in the presence of high grain counts are not critical for cell identification). This extended dynamic grain count range may be useful in the detection of broader ranges of relative rates of DNA synthesis in individual cells, in comparing mean grain counts/cell among different populations, and in following multiple sequential cell divisions in grain count halving studies.

It is evident that there is considerable scatter in the data in the region above 100 grains/cell. This may be due to several factors. First, equation 6 assumes that grains are distributed randomly above the cell nucleus; in fact, grain clustering is common, especially in heavily

labeled cells. Second, the properties of equation 6 are such that the correction for grain overlap is negligible in large cells with low grain counts, and that the correction is of considerable magnitude in small cells with high grain counts. When the total grain area represents a large fraction of the total nuclear area, small differences in total grain area imply relatively large changes in the correction for grain overlap. Thus, in very heavily labeled cells, threshold effects in the grain area measurement become important and may contribute to the variability of the corrected grain count measurement. Finally, in very heavily labeled cells, inaccuracy of the reference visual grain count may also contribute to the apparent variability in the corrected grain count. For some purposes (e.g., determination of mean grains/cell, and grain count halving studies), this variability is not critical. In studies where such variability could be problematic, it might be preferable to restrict the dynamic range of the grain count distribution by appropriate adjustments of ^{3}HTdR concentration and/or emulsion exposure duration.

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FIGURE LEGENDS

Figure 1. Cell-by-cell comparison of grain counts obtained from grain area measurements _without_ correction for grain overlap (ordinate) and visual grain counts (abcissa). For discussion, see text.

Figure 2. Cell-by-cell comparison of grain counts obtained from grain area measurements _with_ correction for grain overlap (ordinate) and visual grain counts (abcissa). For discussion, see text.

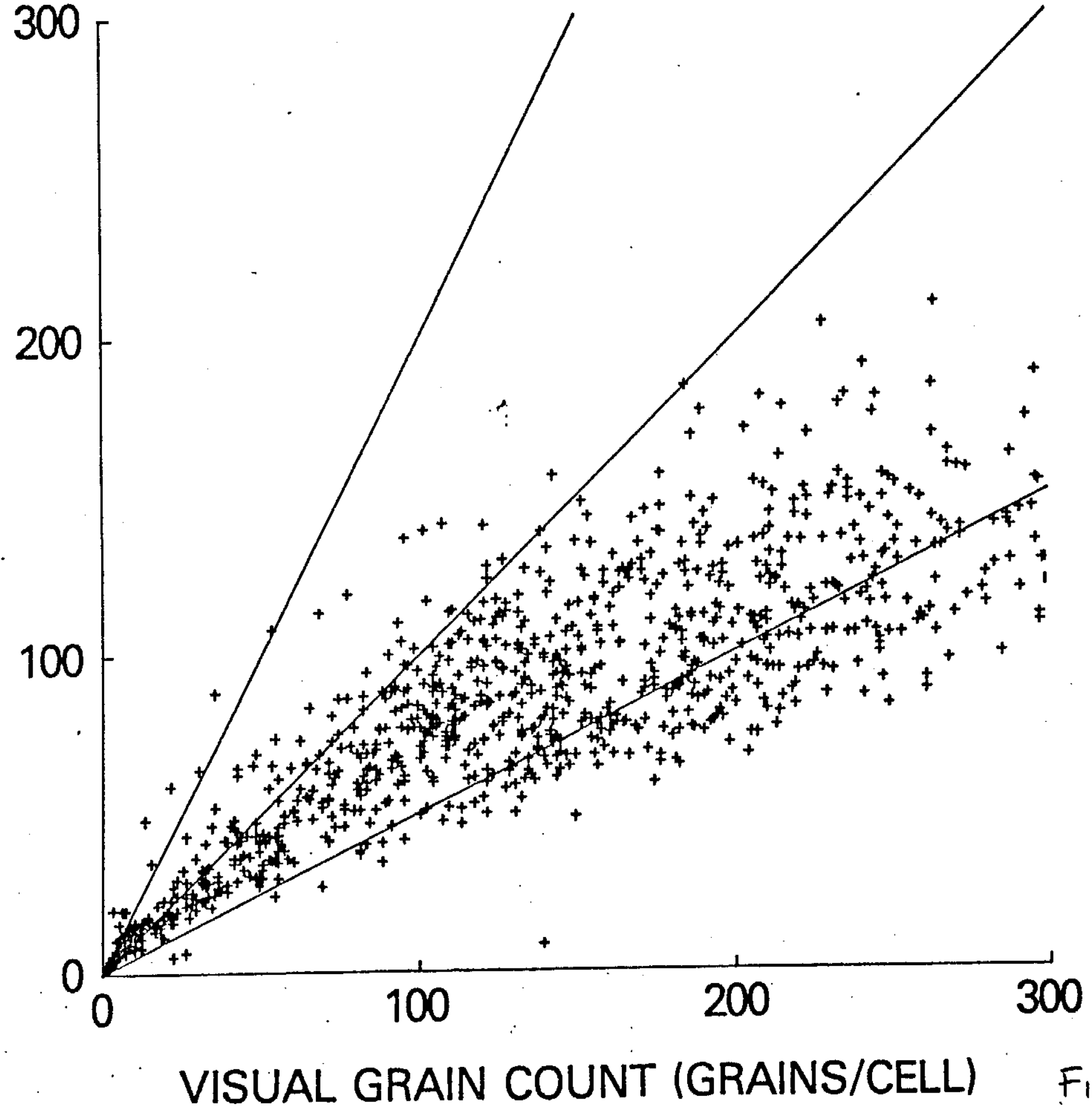

Fig 1

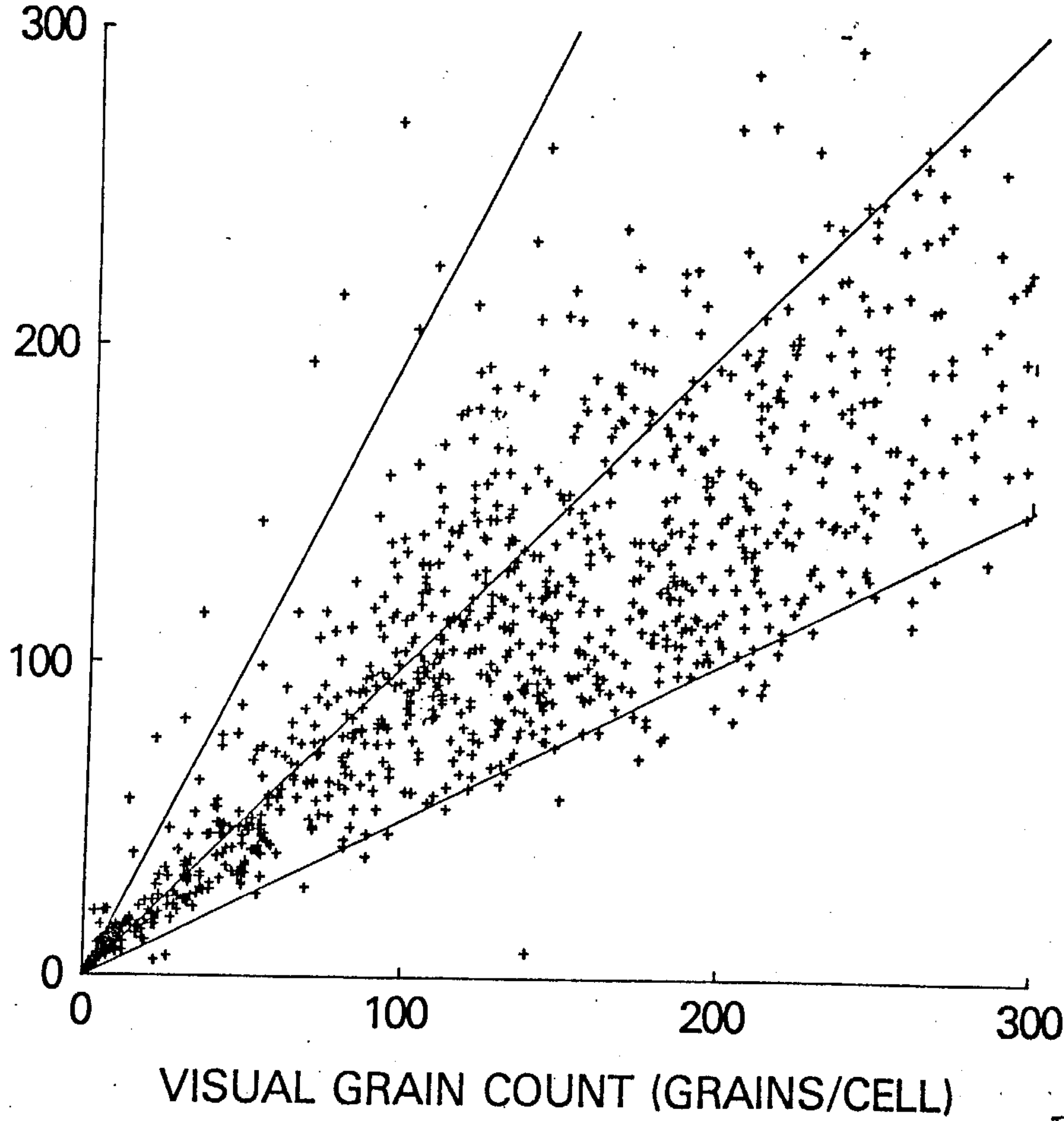

Fig 2