A NEW METHOD FOR ANALYZING

ELECTRON MICROSCOPE AUTORADIOGRAPHS USING

HYPOTHETICAL GRAIN DISTRIBUTIONS

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ABSTRACT

A new method for the analysis of electron microscope autoradiographs is described which has two advantages over other methods. This method provides estimates for the activity in different regions of the section and takes into account the actual geometrical shape and position of different regions within the section. Use is made of the measured distribution of grains about a thin line source to construct a hypothetical distribution of autoradiographic grains which is then compared with the actual distribution of grains. Different activities are then ascribed to various regions until a satisfactory agreement with the autoradiographic grains is obtained using the χ^2 test for significance. In the next paper, an example of the use of this method is given for the distribution of grains in the nucleus of erythroid bone marrow cells labeled with radioactive iron, ^{55}Fe.

INTRODUCTION

A new method for analyzing electron microscope autoradiographs has been devised which combines the "circle analysis" of Williams (1969) with the mathematical considerations of resolution reported by Salpeter et al. (1969).

The method described offers two distinct advantages over previous methods. It takes into account the particular shapes and sizes of the structures observed in the autoradiographs being used, and so avoids the need for constructing theoretical models of subcellular structures. It also provides estimates for the activity within different structures consistent with the distribution of autoradiographic grains.

The method is first described for a simple model situation of a cell containing nucleus, mitochondria, granules, endoplasmic reticulum, and groundplasm. Further aspects of the method are then discussed. In the accompanying paper the method is applied to the distribution of radioactive iron (^{55}Fe) in the nucleus of erythroid cells.

The method, in outline, involves using the experimentally observed distribution of grains about a thin line source of the isotope employed, to calculate the distribution of grains about a point source for the particular section and emulsion thickness used in the autoradiograph as described by Salpeter et al. (1969). This distribution is then used to construct a hypothetical distribution of grains over the autoradiographs being analyzed, assuming a certain amount of activity in the various structures of the autoradiographs. The hypothetical grain distribution is then compared with the real distribution of grains and the activities in the various structures changed until a statistically good fit is obtained, using the χ^2 test of significance. In practice, the method is very easy to carry out.

Description of Analysis Using Hypothetical Grains

A grid of points is, first of all, superimposed over the autoradiographs, and each point is considered to be the site of a radioactive disintegration.

The position at which grains might be produced by these disintegrations is then obtained by making use of a list of "distances" and "directions." These distances are obtained on a random basis such that the frequency with which any distance occurs in the list is equal to the probability of a disintegration from a point source producing a grain at this distance. The method of calculating these distances is explained in the Appendix.

Fig. 1 shows an idealized cell with a superimposed grid of points and hypothetical grains which are joined to the site of disintegration by a line. Since the grid points lie uniformly over the section the hypothetical distribution of grains, at this stage, is for uniform activity throughout the section. This distribution will be considered first before adjustment is made for different activities in the various structures.

Fig. 1 gives some indication of the "cross fire" between adjacent areas and this can be quantitated by applying circles as in the circle analysis method of Williams.

This involves centering a circle on each grain and recording the structure or combination of structures which lie within the circle. An example
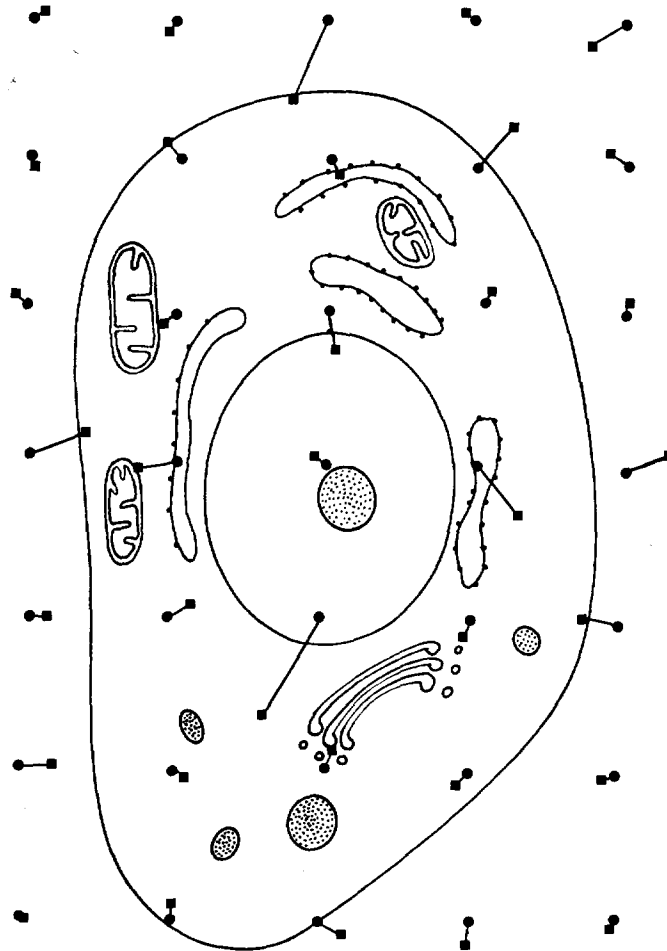

FIGURE 1 An idealized cell with a superimposed regular grid of points (●) and hypothetical grains (■) joined by a line to the site of disintegration.
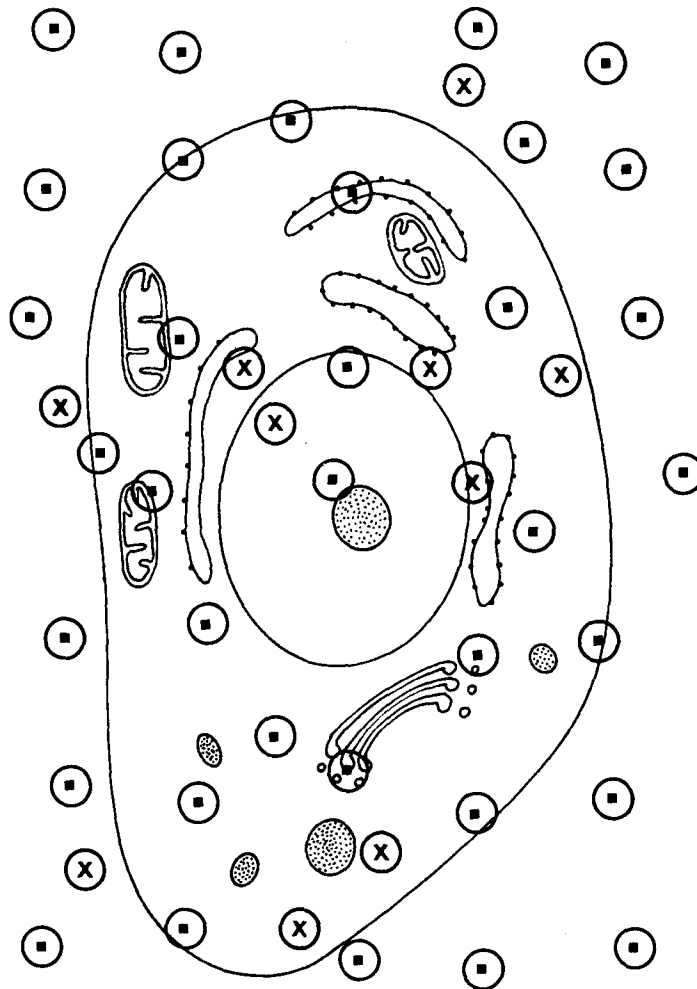
FIGURE 2   The idealized cell with the same distribution of hypothetical grains (■) as in Fig. 1, together with real grains (×). Circles of radius HR are drawn around all the grains.

is shown in Fig. 2 for hypothetical and also real grains. The size of this circle depends on the isotope and the experimental conditions as discussed later. The result of using a grid of 500 points on the model cell is given in Table I.

Each row represents the numbers of grains arising from disintegrations occurring in the region indicated in the column on the left which have circles lying over the regions shown at the top of each column.

The total number of grains in each row shown at the right-hand side represents the total activity in the region concerned and in this case is proportional to the relative area of the structure (since the distribution of activity has for the

moment been assumed to be uniform). The amount of cross fire is shown by the distribution of grains along the rows. The total number of grains in each column gives the circle analysis for this uniform distribution of isotope.

Table I can be modified very easily to obtain a distribution of hypothetical grains for different activities in the different regions. Suppose it was postulated that the activity in the granules was twice that in the other regions and that there was no activity in the mitochondria. All that is necessary is to multiply the number of grains in the fourth row by a factor of two and put all the values in the third row to zero as shown in Table II. There is no need to reapply the grid points.

The total number of grains in each column now gives the hypothetical distribution of grains for this distribution of activity.

If a circle analysis is now carried out on the real autoradiographic grains shown as crosses in Fig. 2, the two distributions can be compared using the χ^2 test for significance, as shown in Table II. The method of calculating χ^2 is described in the following paper. The value of χ^2 for each column demonstrates a reasonably good fit,

and this is confirmed in the total χ^2 value. The statistical probabilities that the two circle distributions are the same are also shown.

In practice, there is no need to mark the hypothetical grains on the autoradiographs as the position and the structure lying under the circle centered on the grain can be obtained in one operation by the use of a small transparent scale. Fig. 3 shows the scale position for grains situated as shown in Fig. 1. The scale is placed so that the

TABLE I

Distribution of Hypothetical Grains for Uniform Activity Throughout the Section

Source of hypothetical grains	Grains per unit area	Site of hypothetical grains											Total grains in each source
		GP	Nuc	Mit	Gran	ER	Golgi	GP/ Mit	GP/ Nuc	GP/ ER	GP/ Gran	GP/ Golgi	
Groundplasm	1	200	5	7	7	0	1	9	7	2	4	2	244
Nucleus	1	4	12	0	0	3	1	0	8	1	0	0	29
Mitochondria	1	3	0	8	1	3	0	6	2	1	2	1	27
Granules	1	16	2	3	45	6	5	3	1	2	25	3	111
Endoplasmic reticulum	1	6	5	3	1	15	2	0	4	30	2	0	68
Golgi	1	4	1	0	1	1	8	0	0	0	1	5	21
Total		233	25	21	55	28	17	18	22	36	34	11	500

TABLE II

Distribution of Hypothetical Grains for Chosen Activities in Various Parts of the Section

| Source of hypothetical grains	Activity per unit area	Site of hypothetical grains											Total activity of source
		GP	Nuc	Mit	Gran	ER	Golgi	GP/Mit	GP/ Nuc	GP/ER	GP/ Gran	GP/ Golgi	
Ground-plasm	1	200	5	7	7	0	1	9	7	2	4	2	244
Nucleus	1	4	12	0	0	3	1	0	8	1	0	0	29
Mito-chon-dria	0	0	0	0	0	0	0	0	0	0	0	0	0
Granules	2	32	4	6	90	12	10	6	2	4	50	6	222
Endo-plasmic reticu-lum	1	6	5	3	1	15	2	0	4	30	2	0	68
Golgi	1	4	1	0	1	1	8	0	0	0	1	5	21
Total		246	27	16	99	31	22	15	21	37	57	13	584
Real grains		201	25	15	80	21	25	17	15	30	48	11	488
χ^2		0.06	0.15	0.25	0.09	1.2	2.9	2.4	0.43	0.03	0.28	0	7.8
Prob-ability		0.98	0.95	0.80	0.98	0.45	0.2	0.25	0.70	0.98	0.80	0	0.6
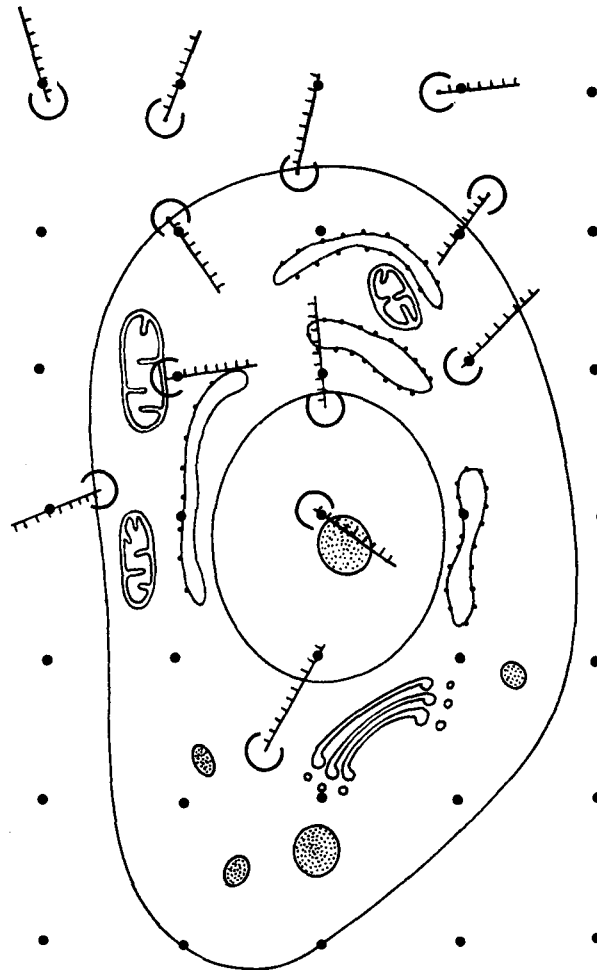
FIGURE 3 Illustration of the use of a transparent circle and scale for determining the structure underlying the site of hypothetical grains. The source is indicated by (O) and the hypothetical grain is at the center of the circle (cf. Fig. 1).

grid point is the required distance from the center of the circle with the scale at the required angle to some arbitrary north-south direction. The structure lying within the circle is then recorded directly.

DISCUSSION

The preceding description of the new method for analyzing electron microscope autoradiographs has been kept intentionally simple in order to demonstrate the basic reasoning behind the method. Further aspects of the method will now be considered.

The use of the circle analysis method of Williams is essential as it provides information on the labeling of linear structures such as membranes which would not be possible if only the position of the grains were recorded. The size of the circle used in the analysis cannot be fixed by any particular criterion. A radius equal to about the "half-distance" for a point source (HR distance) is suitable as suggested by Williams (1969). Smaller circles will miss too many grains originating from membranes, and larger circles will give rise to too many junctional regions.

In addition to ascribing activity to different areas in the autoradiographs, it is possible to include activity in membranes or other line sources. This cannot be done with the grid points because very few will fall over a membrane. In-

stead, points are marked along the membrane (with any convenient spacing), and these are considered to be sites of disintegration of the isotope. The circle and scale is applied exactly as for the points of the grid and entered into the table and adjusted with the other rows to fit the distribution.

It has already been explained how the number of grains in each row is varied in trying to fit the real distribution of grains. The total number of grains in the row represents the total activity in the region. The factor by which the original number of grains has been multiplied is proportional to the activity per unit area, i.e., 0 for mitochondria, 1 for groundplasm, 2 for granules (Table II), and for a membrane is proportional to the activity per unit length in the section.

The choice of activity in different regions of the autoradiographs is to some extent arbitrary since any distribution is possible that gives a sufficiently good fit on the basis of the χ^2 test, that is, with probability values of 0.05 or more. It is therefore necessary to explore the range of possible activities which are consistent with a good fit. The number of regions considered as being labeled is also important since the more regions considered the easier it will be to fit the experimental grains but the greater will be the range of possible activities. An example of the range of values in one particular situation will be found in the following paper for the distribution of ^{55}Fe in the nuclei of erythroid cells.

Ideally, autoradiographs of a thin line source should be made using the same section thickness, emulsion, and development conditions as employed for the autoradiographs being analyzed, and the complete curve used to calculate distances. However, in practice it is probably acceptable to use values of the half-distance (HD) of a line source for similar experimental conditions published by other workers, and then use the formula HR = 1.7 × HD (Salpeter et al., 1969). The distribution of grains from line sources and HD values for sections of different thicknesses and for different emulsions have been published by Salpeter et al. (1969, 1971) for the isotopes ^3H and ^{14}C, the latter being also applicable to ^{35}S due to the similarity in β energy. Data for ^{55}Fe is given in the following paper.

Using this data, lists of grain distances and directions have been calculated for a point source on a random basis and are available on request.

For convenience, these values have been given for a HR of 1,000 Å and a magnification of 10,000. For other HR values and magnifications, the distances must be multiplied by $\dfrac{HR}{1,000}$ and by $\dfrac{magnification}{10,000}$.

The way in which the list of distances and directions, used for locating the hypothetical grains, are obtained has been confined in an Appendix for the benefit of the nonmathematical reader and because it follows very closely the analysis of Salpeter et al. (1969). In the following paper, this new method of analysis is applied to the distribution of grains in autoradiographs of erythroid bone marrow cells labeled with radioactive iron, showing how it provides an estimate for the activity located at the boundary between the condensed and extended nuclear chromatin.

APPENDIX

To calculate the distance and direction of hypothetical grains it is necessary to know the distribution of grains about a point source for the particular section and emulsion thickness and the type of emulsion and development conditions, as they all influence this distribution.

Point sources cannot be constructed for autoradiography, and Salpeter et al. (1969) have shown how the need for such sources can be circumvented by using thin line sources which are relatively easy to make.

A theoretical equation is first established for the distribution of grains about a point source based on geometrical considerations of section and emulsion thickness but excluding consideration of the energy spectrum or scattering of the particles and the size of the developed grains. The distribution about a line source is then calculated and fitted to the experimental data. The distribution about a point source can then be obtained.

In practice, the equations for the distribution of grains about a line source are modified to give a better fit to the experimental results, and this provides an empirical means of taking into account the energy spectrum and scattering of the particles in the section and also the grain size.

The following analysis is essentially that used by Salpeter et al. (1969), except that a different modification is used to improve the fit to the experimental data that we had available for the radioactive isotope of iron, ^{55}Fe.

The proportion of grains, F_p, lying within a circle centered over a radioactive point source is:

$$F_p = 1 - \frac{1}{\left(1 + \frac{x^2}{d^2}\right)^{1/2}}, \quad (1)$$

where x is the radius of the circle and d is the distance between the midplane of the section and the midplane of the emulsion. F_p is merely the solid angle subtended by a circle of radius x at a point distance d away.

This equation can be differentiated, with respect to x, to give the probability that a grain is found at a distance x from a position directly above the disintegration. Differentiating and normalizing so that

$$\int_0^\infty P_p(x) \, dx = 1 \quad \text{gives} \quad P_p(x) = \frac{xd}{(d^2 + x^2)^{3/2}}. \quad (2)$$

$P_p(x)$ is the distribution of grains about a point source. $P_p(x)$ is now divided by $2\pi x$ to give the grains per unit area at a distance x and then integrated to obtain the grains per unit area at a distance from a line source. The integration is from $y = -\infty$ to $y = +\infty$,

$$P_l(x) = \frac{d}{2\pi} \int_{-\infty}^{+\infty} \frac{dy}{(d^2 + x^2 + y^2)^{3/2}}, \quad (3)$$

gives after normalizing so that

$$\int_0^\infty P_l(x) \, dx = 1,$$

$$P_l(x) = \frac{2d}{\pi(d^2 + x^2)}. \quad (4)$$

In practice, it is more convenient to fit the experimental data if it is plotted as the proportion of grains which have a range less than a certain distance. This requires integration of Equation 4 giving:

$$P_l{}^1(x) = \frac{2}{\pi} \tan^{-1}\left(\frac{x}{d}\right). \quad (5)$$

Salpeter et al. found that this curve does not fit the experimental data for tritium particularly well and they added an extra term. We have also found this for ^{55}Fe but have used a different additional term namely:

$$P_p(x) = \frac{2ax}{(d^2 + x^2)^{3/2}} + \frac{2(1-a)c^2x}{(c^2 + x^2)^2}. \quad (2')$$

Integrating as already described gives:

$$P_l{}^1(x) = \frac{2a}{\pi} \tan^{-1}\left(\frac{x}{d}\right) + \frac{(1-a)x}{(c^2 + x^2)^{1/2}}. \quad (5')$$

This equation is used in the following paper to fit the experimental data for a thin line source.

Having therefore obtained an equation for the distribution of grains about a point source from a knowledge of the distribution about a line source, the next step is to use this equation to predict on a random basis the distance of grains from a point source.

Random Selection of Distances

This can be done by means of random numbers. A value of x is chosen at random and $P_p(x)$ is calculated using Equation 2'. A random number in the range 0–1 is then chosen, and if its value is less than the value of $P_p(x)$ it is concluded that a grain has been produced at this distance. If the value is greater than $P_p(x)$, a new value of x is obtained and the process repeated. In this way a list of values of x is obtained such that the frequency of any value of x is given by $P_p(x)$. These distances can be conveniently generated by computer.

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