A NOVEL PROCEDURE FOR PATTERN ANALYSIS OF FEATURES PRESENT ON FREEZE-FRACTURED PLASMA MEMBRANES

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SUMMARY

We have used statistical methods for the analysis of two-dimensional point patterns to derive quantitative descriptions of the distributions of caveolae on freeze-fractured muscle fibre membranes. One method was based on a quadrat analysis while the second was a new procedure that we have called the interpoint distance analysis. We show that the latter analysis can unambiguously distinguish random, clustered and dispersed patterns and that a single parameter can be derived that can be used to compare different distributions. It is readily applicable to patterns containing several hundred points. Practical details of the method are given and a simple algorithm that can be implemented on a microcomputer is provided. The interpoint distance analysis should prove generally useful in situations where the two-dimensional distribution of objects has to be quantified.

INTRODUCTION

A large amount of information is present in electron micrographs of freeze-fractured membranes, but there are difficulties in deriving numerical descriptions of the distributions of features in such images apart from their densities. We are interested in the plasma membrane of muscle fibres from patients with Duchenne muscular dystrophy (DMD), a disorder in which abnormalities of the plasma membrane have been described in a variety of cells (Rowland, 1980; Jones & Witkowski, 1983), including altered morphology revealed by freeze-fracture (Shotton, 1982). Amongst the features of the skeletal muscle membrane revealed by freeze-fracture are caveolae, small surface invaginations of the sarcolemma, and Bonilla, Fischbeck & Schotland (1981) reported an altered distribution of these in DMD muscle fibres. This took the form of a reduced number of fibres showing a large-scale banding of caveolae. In the course of analysing smaller-scale distributions of caveolae, we became aware that simple, visual inspection of these patterns was unsatisfactory and we were led to examine the various quantitative analyses that have been used to describe such patterns.

Quantitative descriptions of the distribution of features such as caveolae can be obtained by treating them as two-dimensional point patterns and applying appropriate

Key words: freeze-fracture, pattern analysis, caveolae, muscle, membrane.
statistical analyses. Problems of pattern analysis arise in many fields and have been studied particularly in relation to the distributions of organisms in ecological research (Kershaw, 1964; Rogers, 1974). Nevertheless, it is not clear what constitutes 'appropriate' analysis for a particular problem (Ripley, 1981) and only two basic types of analysis appear to have been applied in freeze-fracture studies. One analysis is based on a comparison of the observed distribution of points within a number of equal sampling quadrats with that expected for a Poisson (random) distribution (e.g. see Pauli, Friedell & Weinstein, 1978), and the second on the radial distribution function, describing the densities of points at increasing distances about each point (e.g. see Gershon, Demsey & Stackpole, 1979). Both analyses have serious limitations (discussed below) and we have assessed two modified methods for spatial point analysis using data on the distributions of caveolae in freeze-fractured plasma membranes of skeletal muscle.

The first method was to analyse their distribution within contiguous quadrats of a given size, and then to repeat this several times using quadrats of increasing unit size each time. A similar procedure has recently been evaluated by de Laat, Tertoder & Bluemiale (1981) and Niedermeyer & Wilke (1982). The second method that we have called interpoint distance analysis (IPD) involves an analysis of the distribution of distances between each point and all other points (Ripley, 1977). (During the preparation of this paper, a similar method was described by Pedro, Carmo-Fonseca & Fernandes (1984).) In this paper we will describe these analyses and critically compare their performance with other methods. We present the details of the IPD analysis in a non-technical form and we give a simple algorithm for its calculation that can be implemented on a microcomputer. Results using the IPD analysis with normal and pathological human muscle will be presented elsewhere.

MATERIALS AND METHODS

Sample preparation

Samples of muscle for freeze-fracture were obtained by needle biopsy of the quadriceps muscle of the left thigh (see Table 1 for details of subjects). The muscle was fixed in 2% paraformaldehyde and 1% glutaraldehyde in 0.1M-cacodylate buffer (pH 7.2) at room temperature for 1 h. After 20–30 min in fixative when further contraction was unlikely, the muscle was cut or teased into small, thin pieces. After fixation, the muscle was washed in buffer (3 changes, 5 min each) to remove fixative and then infiltrated with increasing concentrations of glycerol (10%, 20% and 30%) in cacodylate buffer, 30 min in each concentration. The tissue was sandwiched between gold alloy or copper double-replica specimen supports (Balzers) and frozen by plunging into liquid Freon 22, which was refreezing in a bath of liquid nitrogen. The samples were then stored submerged in liquid nitrogen.

Freeze-fracture

The samples were transferred, still under liquid nitrogen, to the double-replica device of a Balzers BAF 301 freeze-etch machine and orientated so that the long axis of the muscle fibres was parallel to the direction of the platinum gun. The chamber was evacuated to a pressure of less than $10^{-9}$ mbar (1 bar = $10^5$ Pa) and the specimen temperature was raised to $-115^\circ$C to minimize condensation of water vapour onto the fractured surfaces and etching or sublimation of water from the surfaces. After fracturing, the specimen surfaces were immediately coated with platinum from a unidirectional
source at an angle of 45° to the surfaces to a depth of 2 nm, followed by a coating of carbon applied from a source at an angle of 75° to the specimen surface while the specimen rotated in the carbon beam. The tissue was digested in 30% sodium hypochlorite for 2-3 h, the replicas cleaned and collected on uncoated 400 mesh copper specimen-support grids. Replicas were examined in a Zeiss EM10 electron microscope operated at 80 kV.

**Digitization of caveolae distributions**

Suitable areas for photography and analysis were selected according to the following criteria: (1) that the area of membrane chosen should approximately fill the negative area of the microscope camera at a magnification of ×6300; (2) that this area of membrane should be relatively free of folds, and relatively flat and horizontal with respect both to the original platinum shadowing and to the electron beam of the microscope. Any fibre that contained an area satisfying these criteria was photographed. Prints were made at a final magnification of ×18,900 and the most satisfactory prints of each of 10 fibres from each subject were selected, giving a total of 50 fibres for analysis.

A suitable area (100 mm × 100 mm corresponding to 5.3 µm × 5.3 µm, 28 µm²) of each print was selected according to the above criteria and the X–Y co-ordinates of all caveolae within this area were recorded with a resolution of 0.1 mm using a Reichert-Jung MOP-1 digitizer interfaced with a CBM 3032 microcomputer. The latter was fitted with a MOPPET ROM (MOPPET Laboratories, London) designed for use with the MOP-1 digitizer (Round, Jones & Edwards, 1982). For convenience, we use mm distances on the final photograph, where 1 mm is equivalent to 530 nm. A program was written for the CBM 3032 for the collection of data from the digitizer and for storing it on floppy discs (CBM 3040 disc drive, 5 1/4 in single-sided, single density discs). On this system, data from up to 30 samples could be stored on one disc (total capacity 170 kb).

**Statistical analyses**

**Quadrat analysis**

The quadrat analysis used is a standard procedure in other fields, e.g. ecological research (Kershaw, 1964). The 100 mm × 100 mm square was divided into smaller squares, ranging from four of 50 mm × 50 mm to 1600 of 2.5 mm (see Table 2 for full range of sizes). The program determined, from the X–Y co-ordinates of the caveolae, the mean and variance of the number of caveolae per square for each size of square. The ratio of variance/mean (the coefficient of dispersion, cd) was then calculated for each quadrat size.

**IPD analysis**

This analysis is based on the work of Ripley (1977, 1981). The program performs the calculation in the following way (Fig. 1). (1) An inner square, 80 mm × 80 mm, was constructed within the sample area so that there was a border 10 mm wide surrounding it. This border is called the guard area and is cross-hatched in Fig. 1. (2) The program checked if a point lay within the inner square or within the guard area. (3) The distances were calculated between each point in the inner square and all other points. (4) Distances between points both lying in the guard area were not calculated. The distance (t) between points was plotted (abscissa) against L(t), proportional to the square root of the number of pairs of points closer together than that distance (Figs 4,7). The plot for a random distribution is a straight line (x = y) passing through the origin, and the curves for clustered and dispersed patterns would lie above and below this line, respectively; 95% confidence limits can be calculated and used to identify
Fig. 1. IPD calculation. The sample area (A) of 100 mm × 100 mm has an inner square (B) of 80 mm × 80 mm constructed within it. Points P1 to P4 lie within the guard area (cross-hatched) surrounding B, and distances between these points are not calculated. The remaining points lie within B, and the distances between each of these points and all other points (including those lying within the guard area) are calculated (———).

those parts of the curves that differ significantly from the line for a random distribution. Details of the analysis and an algorithm for its calculation from the X and Y co-ordinates are given in the Appendix.

Figs 2–4. Subject D.M. Fig. 2, area of freeze-fracture micrograph analysed; Fig. 3, positions of caveolae digitized are marked and the guard area indicated; Fig. 4 IPD analysis of the caveolae marked in Fig. 3. The 95% confidence limits are marked (·····) and the unbroken line is the plot expected for a random distribution. The distance of maximum regularity is marked (arrowhead, $t = 12$).

Figs 5–7. Subject A.B. Fig. 5, area of freeze-fracture micrograph analysed; Fig. 6 positions of caveolae digitized are marked and the guard area indicated; Fig. 7, IPD analysis of the caveolae marked in Fig. 3. The 95% confidence limits are marked (·····) and the unbroken line is the plot expected for a random distribution. The distance of maximum regularity is marked (arrowhead, $t = 27$).
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Figs 2-7
Computation

Programs for statistical analysis were written in Basic and run on a CBM 8032 microcomputer. This is the standard microcomputer used in the Muscle Research Centre and is a low-cost but relatively low-performance machine. Analyses were also performed on the BBC microcomputer that is widely used in scientific laboratories. In addition, because the IPD analysis requires the calculation of a large number of values, we were interested to determine the improvements in speed attainable using faster machines than microcomputers. For this purpose, the IPD analysis was also performed on a Perkin-Elmer PE3220 'super' minicomputer and an Amdahl 470V/8 mainframe computer.

Test patterns

The random, clustered and dispersed patterns published by Niedermeyer & Wilke (1982) were used with our quadrat and IPD analyses. These patterns are 50 mm × 50 mm and a guard area of 5 mm was used. We did not distinguish between the two sizes of object present in these patterns and treated them as points. These patterns are shown in Figs 8–10.

RESULTS

Caveolae densities

Typical examples of the photographs analysed in this study are shown in Figs 2 and 5, and the positions of caveolae marked during digitization are shown in Figs 3 and 6. The sample squares (100 mm × 100 mm) contained on average 528 caveolae, corresponding to an overall mean density of 18.9 caveolae/μm. The caveolae densities for each subject are given in Table 1. There were small variations in density between subjects but these were swamped by considerable density variations of over 2:1 between fibres, while fibres from subject A.B. had more consistent caveolae densities over a range of only 1:25:1. With such large variations in density, it was possible that the spatial interactions described below were dependent on caveolae density. This was investigated but no such dependence was found.

Quadrat analysis of caveolae distributions

The program determined the variance/mean ratio of caveolae densities over a series of 10 quadrat sizes. If the patterns of caveolae were random then the counts of caveolae would be statistically independent samples from the Poisson distribution, and variance/mean ratios near unity would be expected; larger values indicate clustering...
Table 1. Caveole densities

<table>
<thead>
<tr>
<th>Subject</th>
<th>Diagnosis</th>
<th>Mean cavole density/μm²</th>
<th>s.d.</th>
<th>No. of fibres</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.M.</td>
<td>Normal</td>
<td>16.5</td>
<td>3.75</td>
<td>10</td>
</tr>
<tr>
<td>D.M.W.</td>
<td>Normal</td>
<td>21.5</td>
<td>6.23</td>
<td>10</td>
</tr>
<tr>
<td>D.S.</td>
<td>DMD</td>
<td>19.9</td>
<td>3.98</td>
<td>10</td>
</tr>
<tr>
<td>M.M.</td>
<td>DMD</td>
<td>18.2</td>
<td>5.14</td>
<td>10</td>
</tr>
<tr>
<td>A.B.</td>
<td>Limb-girdle dystrophy</td>
<td>18.4</td>
<td>1.19</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2. Quadrat analysis of caveole distributions

<table>
<thead>
<tr>
<th>Quadrat* unit (mm)</th>
<th>Subject D.M.; fibre 8715</th>
<th></th>
<th>Subject A.B.; fibre 7317</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean†</td>
<td>Variance</td>
<td>cd‡</td>
<td>Mean†</td>
<td>Variance</td>
</tr>
<tr>
<td>50</td>
<td>138.5</td>
<td>93.66</td>
<td>0.676</td>
<td>128.3</td>
<td>200.24</td>
</tr>
<tr>
<td>33.3</td>
<td>61.6</td>
<td>13.27</td>
<td>0.215</td>
<td>57.0</td>
<td>31.24</td>
</tr>
<tr>
<td>25</td>
<td>34.6</td>
<td>36.11</td>
<td>1.043</td>
<td>32.6</td>
<td>14.72</td>
</tr>
<tr>
<td>20</td>
<td>22.2</td>
<td>26.22</td>
<td>1.183</td>
<td>20.5</td>
<td>10.84</td>
</tr>
<tr>
<td>16.6</td>
<td>15.4</td>
<td>17.44</td>
<td>1.133</td>
<td>14.3</td>
<td>10.76</td>
</tr>
<tr>
<td>12.5</td>
<td>8.7</td>
<td>10.51</td>
<td>1.214</td>
<td>8.0</td>
<td>4.30</td>
</tr>
<tr>
<td>10</td>
<td>5.5</td>
<td>5.20</td>
<td>0.938</td>
<td>5.1</td>
<td>2.17</td>
</tr>
<tr>
<td>5</td>
<td>1.4</td>
<td>1.13</td>
<td>0.819</td>
<td>1.3</td>
<td>0.63</td>
</tr>
<tr>
<td>2.5</td>
<td>0.3</td>
<td>0.28</td>
<td>0.820</td>
<td>0.3</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Overall sample area was 100 mm x 100 mm (equivalent to 5.3 μm x 5.3 μm), and this was divided into units of the sizes given in this column.
† Mean is the mean number of caveolae per quadrat unit.
‡ Coefficient of dispersion is given by variance/mean. Values greater than unity indicate aggregation and values less than unity indicate dispersion of caveolae.

( aggregation) of points and smaller values indicate regularity (dispersion) (Ripley, 1981; Rogers, 1974). Examples of the analyses of one sample area of one muscle fibre from each of subjects D.M. and A.B. are given in Table 2.

All the samples showed regularity at the smallest scale (2.5 mm quadrat equivalent to 130 nm), as would be expected from the size of the caveolae (diameter approx. 50–100 nm), but at other scales there was no consistency between fibres from the same individual. Moreover, aggregation and dispersion were sometimes detected at slightly different quadrat sizes within the same sample area. However, one subject (A.B.) had consistent cd values at most quadrat sizes on most fibres tested.

Interpoint distance analysis

Figs 4 and 7 illustrate the plots obtained by an interpoint distance analysis of the caveolae patterns of the same fibres of subject D.M. (Fig. 2) and subject A.B. (Fig. 5) that were analysed by the quadrat method. The abscissa is the distance between pairs of points and the ordinate is proportional to the square root of the cumulative number of
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Table 3. Ordered distances of maximum regularity derived from IPD plots

<table>
<thead>
<tr>
<th>Subject</th>
<th>Distances (× 0.1 mm)</th>
<th>Mean</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.M.</td>
<td>12,12,12,12,13,17,18,18,20,23</td>
<td>15.7</td>
<td>15.0</td>
</tr>
<tr>
<td>D.M.W.</td>
<td>10,11,11,11,11,12,12,14,14</td>
<td>11.7</td>
<td>11.0</td>
</tr>
<tr>
<td>D.S.</td>
<td>6, 8, 8, 9,10,10,12,12,16</td>
<td>10.1</td>
<td>10.0</td>
</tr>
<tr>
<td>M.M.</td>
<td>10,13,14,16,16,17,18,19,21,22</td>
<td>16.6</td>
<td>16.5</td>
</tr>
<tr>
<td>A.B.</td>
<td>23,24,24,25,25,25,26,26,27,28</td>
<td>25.2</td>
<td>25.0</td>
</tr>
</tbody>
</table>

The positions of caveolae in an area of 28μm² on each of 10 muscle fibres from each subject were digitized and analysed. The distance of maximum regularity is the interpoint distance at which the curve (Figs 4, 7) was the furthest below the diagonal line. The individual value for each area analysed is given.

The aim of the work reported in this paper was to assess the interpoint distance method for the analysis of point patterns in freeze-fracture images and to compare it with a method based on the use of quadrats.

Quadrat analysis, with calculation of coefficient of dispersion, is the most frequently used method of spatial analysis in this field (see, e.g., de Laat et al. 1981; Niedermeyer & Wilke, 1982; Pauli et al. 1978). But it is not a satisfactory method for spatial
analysis of point patterns (Ripley, 1981). For example, the positioning of the quadrat grid relative to the points can influence what pattern is detected and the pattern detected also depends on the size of the quadrats used. It can be shown that in a clustered population the distributions detected would be random, clustered and regular as the quadrat size is increased. Most analyses of the distributions of IMPs so far reported have used a single quadrat size and are subject to both of these criticisms. This problem can be mitigated somewhat by using, as we have done, a series of contiguous quadrats of different sizes, and detecting the scales of distance at which significant deviations from a Poisson distribution occur. Niedemeyer & Wilke (1982) have used a series of overlapping quadrats of different sizes but there are statistical errors in their treatment of this problem. Most seriously, this overlapping means that counts that are assumed to be independent cannot be. In addition, their weighted analyses have no statistical foundation and do not have the claimed distributions.

However, even when departures from randomness are detected, it is often difficult to relate these to features in the pattern. Where the pattern detected varies with quadrat size it is difficult to summarize the pattern or to decide on a single value that can be used for comparison between analyses. As different positioning of the quadrats also produces different results, even comparing values for a single quadrat size may not be valid. The logical abstraction of the quadrat method (to consider counts with all possible quadrats of all possible sizes) has been developed (Jolivet, 1978; Liebtrau & Rothman, 1977) and proves to be very similar to the interpoint distance analysis but less easy to interpret.

The interpoint distance analysis is not limited by the choice of an arbitrary scale of distance, as are quadrat methods and those based on the radial distribution function, and it gives plots that can be clearly interpreted. Furthermore, a single value, the interpoint distance of maximum deviation from random, can be easily derived and used to compare different plots. That this analysis can distinguish random, dispersed and clustered patterns is demonstrated by our analysis of the test patterns of Niedemeyer & Wilke (1982) (Figs 8–13).

Given the X–Y co-ordinates of the points, the calculation of an IPD analysis is straightforward. We chose the simplest form of edge correction because a large number of points were available for analysis and the running time of the program was an important factor because a microcomputer was used for the analysis. Typically, an analysis of a sample containing a total of 500 caveolae with 170 in the guard area requires the calculation of about $1 \times 10^3$ IPDs. We used a small microcomputer for these analyses because we were keen to devise a procedure that would run on systems that might be readily available in other laboratories. However, considerable savings in computing time can be achieved if larger machines are available. For example, while for a typical analysis of 500 caveolae, the algorithm given in appendix A took several hours on a CRBM8032 and 40 min on a BBC microcomputer, it took 14 min in Basic and 1·4 min in Fortran running on the PE3220 'super' minicomputer, and 1·4 s on an Amdahl 470V/8. However, as these analyses were run in batches overnight, the slowness of the microcomputer did not prove a limiting factor compared to the time taken for the morphological work and for digitization of the caveolae distributions.
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The IPD analysis is related to the radial distribution function used initially by Markovics, Glass & Maul (1974) for the analysis of nuclear pore patterns, and subsequently by Perelson (1978) for the analysis of the distribution of lymphocyte surface immunoglobulin, and Gershon et al. (1979) for pattern analysis of IMPs. We have also applied the radial distribution function to our samples but found it difficult to interpret the plots obtained (Appleyard & Witkowski, unpublished observation). The IPD analysis has the advantage of a cumulative plot that is statistically more sensitive and is not dependent on the choice of bin width. The distribution theory of $L(t)$ is also better understood than that of the estimate of the radial distribution function used by Markovics et al. (1974).

In some samples the caveolae were found to lie in bands transverse to the long axis of the muscle fibre. This banding also needs to be described, and its analysis may necessitate the development of new statistical theory.

The IPD analysis has proved valuable for the analysis of the small-scale distribution of point objects on samples where no pattern was discernible by eye. We believe that the IPD analysis described here has advantages over previously used methods and should find wide application for describing the two-dimensional distributions of point features in morphological research.

We are very grateful to Dr M. J. Dunn for the Fortran programming. This work was supported by grants from the Wellcome Trust and the Muscular Dystrophy Group of Great Britain.

APPENDIX

For a full discussion of the basis of the inter-point distance analysis, see Ripley (1977, 1981). To allow for edge effects, a central 80 mm $\times$ 80 mm square B was considered within the 100 mm $\times$ 100 mm square A, and the area within A but outside B constituted the guard area (see Fig. 1). Then any point within 10 mm of a point in B must be in A. (More sophisticated and efficient means of correcting for edge effects are available (Ripley, 1981) but the present method was considered sufficient, given the large numbers of points for analysis and the computational limit imposed by the use of a slow microcomputer.) The distances between each pair of points were recorded, except where both points lay in the guard area. Let $n(t)$ be the number of these distances less than $t$. The plots are of $L(t)$ versus $t$, where

$$L(t) = \sqrt{(2A/\pi mn)} \times \sqrt{n(t)}$$

and $A$ is the area of the square (in our case $10^4$ mm$^2$), $n$ is the total number of points, $m$ is the number of points in B. Then for a random pattern of points, the mean of $L(t)$ is very close to $t$ for $t < 10$ mm, and the variance of $L(t)$ is approximately independent of $t$. Finally, if

$$L_{\text{max}} = \max_{0 < t < T} |L(t) - t|$$

is the maximum deviation of $L(t)$ from $t$, where $T$ is the largest distance to be considered, then

$$P(L_{\text{max}} < 1.43 \sqrt{(A/mn)}) \approx 95\%$$
provided $nT / \sqrt{A}$ is in the range 2–25 and $T$ is less than the size of the border, in our case 10 mm.

The following program outlines an efficient algorithm to calculate $L(t)$ at $K$ equally spaced points in $0 < t < T$.

Store $X$ and $Y$ co-ordinates in arrays $x$ and $y$
Zero an array $c$ of $K$ counts
Let $f = K / T, TT = T \times T$

For $i = 2$ to $n$
  If $(x(i), y(i)) \epsilon B$ then $v = 1$ else $v = 0$
  For $j = 1$ to $i - 1$
    If $(x(j), y(j)) \epsilon B$ then $v = v + 1$
    If $v = 0$ then next $j$
    $xh = x(i) - x(j)$
    $yh = y(i) - y(j)$
    $d = xh \times xh + yh \times yh$
    If $d > TT$ then next $j$
    $h = \text{int}(1 + f \times \sqrt{d})$
    $c(h) = c(h) + v$
  Next $j$
Next $i$

$c1 = 0, c2 = \sqrt{2 \times \text{area} / \pi mn}$

For $h = 1$ to $K$
  $c1 = c1 + c(h)$
  $L(h) = c2 \times \sqrt{c1}$
  $t = h / f$
  Print $t, L(h)$
Next $h$

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