Quantitative Microscopic Analysis of Histological Sections of Brain Tissue

R. A. Armstrong*

Vision Sciences, Aston University, Birmingham B4 7ET, UK

This article reviews methods for quantifying the abundance of histological features in thin tissue sections of brain such as neurons, glia, blood vessels, and pathological lesions. The sampling methods by which quantitative measures can be obtained are described. In addition, methods are described for determining the spatial pattern of an object and for measuring the degree of spatial correlation between two or more histological features.

Keywords: histological features, neurodegenerative disease, quantitative measurements, sampling methods, spatial pattern, spatial correlation.

1. Introduction

The last decade has seen a considerable increase in the application of methods designed to quantify objects visible in histological sections of brain tissue [1,2]. Image analysis systems have enabled histological features to be captured and enhanced on a computer screen so that they can be quantified rapidly and objectively [3].

Several features are visible in histological sections including cell bodies of neurons and glial cells, blood vessel profiles, and the discrete lesions that are formed in the tissue as a result of pathological processes. In many neurodegenerative diseases, intracellular inclusions such as neurofibrillary tangles (NFT), Lewy bodies (LB), or Pick bodies (PB) may be observed within the cytoplasm of neurons while extracellular protein deposits of β-amyloid (Aβ) or prion protein (PrP) occur in Alzheimer's disease (AD) and Creutzfeldt-Jakob disease (CJD) respectively. Data on the relative abundances of these lesions are routinely used in the pathological diagnosis of disorders [4] and in studies of disease pathogenesis [5].

This article reviews: 1) sampling methods by which quantitative measures can be obtained from histological sections, 2) methods of quantifying the abundance of an object, 3) methods for determining the spatial pattern of an object, and 4) methods for measuring the degree of spatial correlation between different histological features.

2. Sampling methods

2.1 Plot/Quadrat sampling (Fig 1)

Plot or quadrat sampling is the most commonly used procedure for obtaining quantitative data from a tissue section. The plots may be rectangular, square, or circular in shape, although the rectangular plot is often regarded as the most efficient method of sampling a two-dimensional surface [6]. Positioning the plot relative to the section may be determined by overlaying a grid or other systematic method or by a standard random procedure to minimize bias [7].

* email: R.A.Armstrong@aston.ac.uk, Phone: +44 1212044102

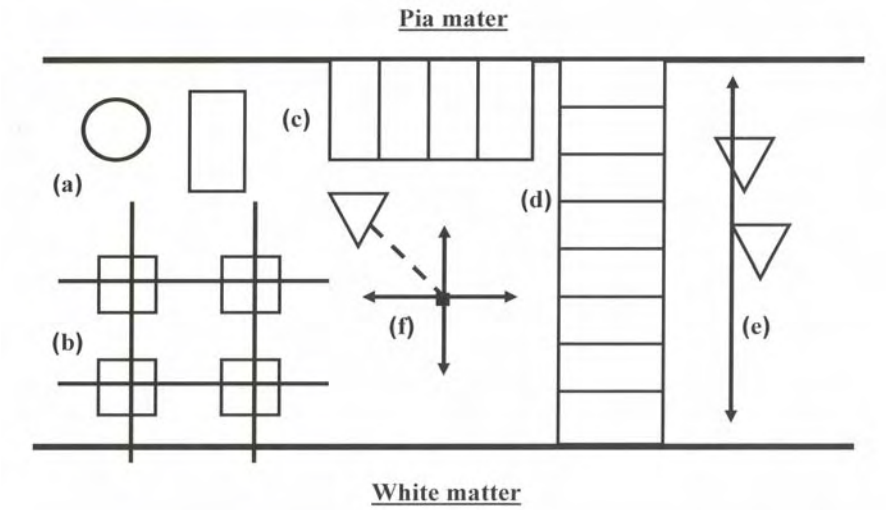

Fig 1. Methods of sampling a section of the cerebral cortex: a) plot sampling with randomly placed circular or rectangular plots, b) plot sampling with square plots arranged in a grid, c,d) belt-transect sampling along and across the cerebral cortex, e) line transect, f) point-quarter sampling.

2.2 Transect sampling

Transect sampling is most appropriate when there is a systematic change in the abundance of an object in a particular direction. A common method of sampling the cerebral cortex, for example, is the use of contiguous, rectangular sample fields that follow the contours of the sulci and gyri (Armstrong 2000). This sampling regime is useful in studying the changes in abundance of pathological lesions that may occur along the cortex parallel to the pia mater. In addition, the method has been extensively used to study changes across the laminae of the cortex [8-11].

Two types of transect sampling can be used. Firstly, in a 'belt-transect', a strip of tissue is sampled in which all objects of interest are counted or measured and is the method most commonly employed [12]. If the transect is divided into contiguous plots, data for all plots can be used to compute a quantitative measure. An example of the use of the ‘belt-transect’ method to study the laminar distribution of PB in the temporal lobe of a case of Pick’s disease (PD) is shown in Fig 2. The distribution of the PB is bimodal with a large density peak in the upper cortex and a smaller peak in the lower cortex. This distribution suggested the involvement of the cells of origin of the feedforward cortico-cortical pathways in PD [13]. Secondly, in the more rarely used line-intercept method, data is tabulated on the basis of the objects that intersect a straight line that cuts across an area to be sampled.

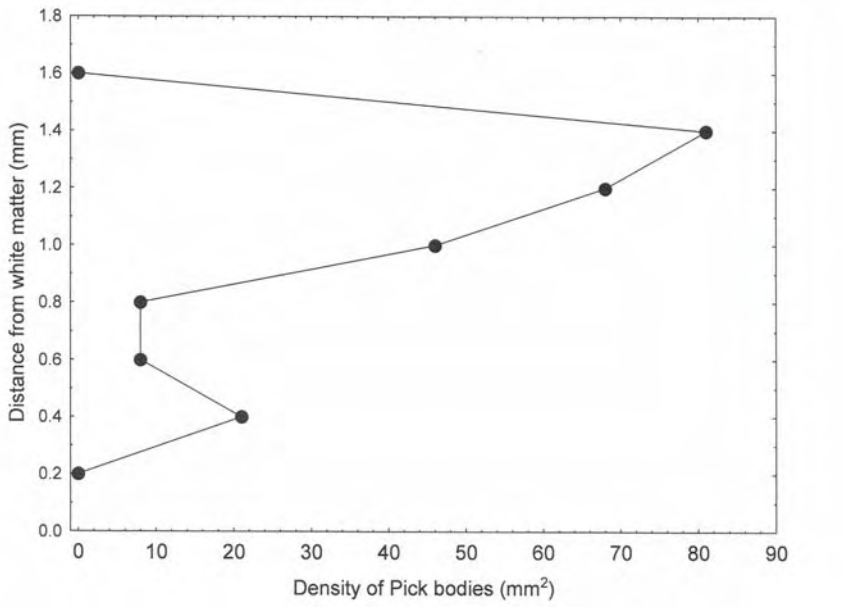

Fig 2. Laminar distribution of Pick bodies (PB) in the lateral occipitotemporal gyrus of a case of Pick's disease (PD).

2.3 Point-quarter sampling

Plot-based methods may be laborious and time-consuming and the results are often dependent on the size, shape, and the number of the plots sampled [5]. By contrast 'plotless sampling' has the advantage of not demarcating sampling areas of a certain size or shape. Plotless methods are sensitive, however, to departures from a random distribution of individual objects especially if the sample size is small [6]. The plotless sampling method of choice is the 'point-quarter method' and is regarded as superior to other plotless methods such as the nearest-neighbour method [5].

A sample of points is established in the study area. These points may be randomly distributed throughout the whole area or along a belt-transect, e.g., parallel to the pia mater. Each point is considered to be the centre of four compass directions dividing the area into four quarters. In each quarter, the distance from the centre point to the nearest object of interest is measured; four objects measured to each point.

3. Measurement of abundance

3.1 Density

Density is the number of individual objects per unit of area. There are two problems in obtaining density measurements in histological sections. First, it may be difficult to define an appropriate area in which the density measurement is to be obtained. A pathological lesion, for example, may develop in relation to the cells of origin of a specific cortical projection and be largely confined to certain cortical laminae [9]. Hence, NFT in AD are frequently found in greater abundance in laminae II/III [11,14]. Depending on distribution, therefore, sampling of an object can be carried out throughout the cortical profile or be confined to particular laminae. Second, it may be difficult to define what constitutes an individual lesion [7]. Many discrete objects such as neurons, glia, or pathological inclusions can be counted successfully by this method. By contrast, it is more difficult to define the boundaries of protein deposits such as

diffuse-type Aβ deposits in AD [15] or the synaptic-type PrP deposits observed in sporadic CJD (sCJD) [8,16]. In circumstances where it is impossible to define an 'individual', alternative measures of abundance such as 'coverage' or 'load' can be used.

3.2 Frequency

Frequency of an object is the number of samples in which a particular type of object is present, e.g., if the object occurred in 7/10 samples, the probability of finding it in an area of tissue would be 0.7 and its frequency 70%. Frequency measurements provide a rapid method of indicating the abundance of an object in a tissue section. Frequency estimates are, however, highly dependent on the size and shape of the plots used. If plots are too large, then it is certain that all types of object, common or rare, will be found in a plot whereas if the plots are too small, then a less common object may be insufficiently recorded. In addition, frequency measurements are sensitive to the distribution pattern of individual objects, i.e., whether the object is distributed at random, regularly, or is aggregated into clusters [5,17]. Many types of lesion in neurodegenerative disorders, for example, exhibit a clustered distribution and sample plots of different size may be necessary to estimate its frequency [5].

3.3 Cover (Mass or load)

Mass or load is the amount of a feature present in the tissue and may be appropriate when measuring less circumscribed lesions such as diffuse Aβ or PrP deposits. Image analysis systems frequently provide estimates of mass or load by measuring 'cover' or 'coverage'. Cover is the proportion of the area of the sample that is occupied by a feature in relation to the total area in which the feature could occur. This method was used to quantify the percentage of tissue occupied by spongiform change in CJD [18,19]. Coverage values can also be obtained by using a sample field divided into a grid and counting the number of times the points of intersection of the grid overlay the object under study. This method has been used to estimate the abundance of diffuse PrP deposits in sCJD [8], Aβ load in AD [7,8,20], and blood vessel profiles in AD [21]. Load is sometimes considered a more useful measure than absolute density in the study of neurodegenerative disease since it may be more closely correlated with clinical symptoms.

3.4 Semi-quantitative scores

A rapid method of describing the abundance of a lesion in a tissue section is to assign a subjective assessment of abundance. For example, the "Consortium to Establish a Registry for Alzheimer's disease" (CERAD) criteria for the clinico-pathological diagnosis of AD [22] scores the abundance of Aβ deposits on a four-point scale, viz., none, sparse, moderate, or frequent. Such a scale can be used not only for diagnostic purposes but also for large-scale studies of many patients and brain areas in which it may not be feasible to obtain more accurate density measurements (Mann et al 1987). This method was used to study the degree of neuropathological heterogeneity within a group of 80 cases of AD (Armstrong et al 2000). The limitations of semi-quantitative scores are the large unconscious error of judgment and the consequent high between-observer variability of the scores.

4. Measuring spatial pattern (Table 1)

Table 1. Formulae and significance tests for studying spatial pattern in histological sections.

Method	Statistic	Significance test	Data
Poisson	V/M	$t = \|V/M-1.0\|/\sqrt{2(n-1)}$	Density
Negative binomial	$1/K$	-	Density
Morisita (I_d)	$I_d = n\,(\sum X^2-N)/$ N(N-1)	$\chi^2 = (n\sum X^2/N)-N$	Density
Holgate (A_1)	$A_1 = \sum (d^2/d_1^2)$ $n-0.5$	$/t = \|A1\|/ (\sqrt{n/12}$	Distance random points to object
Hopkin (A_2)	$A_2 = \sum d^2/\sum d_1^2 - 1$	$t = 2\|\ (A2+1)/(A2+2$ $0.5\|\sqrt{(2n-1}$	)Point to object Object to object Distance

Abbreviations: V = variance, X = individual observations, M = mean, K = binomial exponent, n = number of observations or plots, N = total number of individuals counted on all 'n' plots, d = distance measure, 't' = Student's 't'.

4.1 The Poisson distribution

Methods based on the Poisson distribution are the most commonly used to measure spatial pattern [14]. Any type of plot sampling can be used to fit a Poisson distribution to data including randomly distributed plots, a transect of contiguous plots, or a grid of plots. If dispersion of individuals is random then the probability (P) that the plots contain 0, 1, 2, 3, ..., n, individuals is given by the Poisson distribution [5]. In a Poisson distribution, the variance (V) is equal to the mean (M) and hence, the V/M ratio is unity. The V/M ratio (Table 1) is an index of spatial pattern, uniform distributions having a V/M ratio less than unity and clustered distributions greater than unity. The significance of departure of the V/M ratio from unity can be tested by a 't'-test or by a chi-square (χ^2) test [6].

A disadvantage of the Poisson method is that the results are markedly affected by plot size. To overcome this problem, if contiguous samples or grid-sampling is used, quantitative measures in adjacent plots can be added together successively to provide the data for increasing plot sizes [5,17]. V/M is plotted at each field size and the resulting graph will indicate whether the clusters of lesions were regularly or randomly distributed and at which scale. A peak indicates regularly distributed clusters of lesions while the field size corresponding to the peak is an indication of the mean cluster size. This method has been used to study the spatial pattern of lesions in several neurodegenerative diseases including NFT in AD [24], Lewy bodies (LB) in dementia with Lewy bodies (DLB) [25], and glial cytoplasmic inclusions (Papp-Lantos lesions) in multiple system atrophy (MSA) [29].

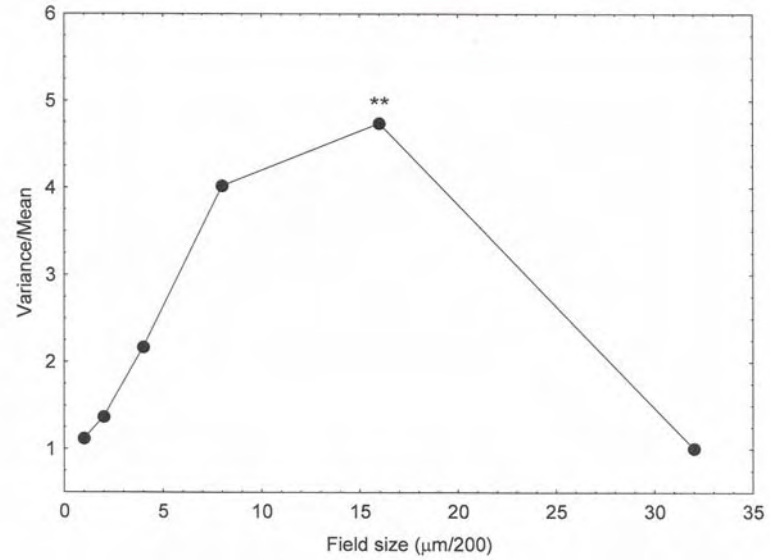

Fig 3. Spatial pattern analysis of Lewy bodies (LB) in the parahippocampal gyrus in a case of dementia with Lewy bodies (DLB) (** significant V/M peak).

An example of the use of this method to study the spatial pattern of LB in the temporal lobe of a case of DLB is shown in Fig 3. Contiguous samples (N = 64), 200 x 1000μm in size, were arranged along cortical laminae V/VI in a belt-transect, the shorter dimension of the sample field parallel to the edge of the white matter. The data show that the V/M ratio increased with field size indicating clustering of the LB and reached a peak at field size 3200μm after which V/M declined. These data suggest the presence of clusters of LB approximately 3200μm in diameter and regularly distributed parallel to the pia mater. This type of spatial pattern is commonly seen in many neurodegenerative disorders [29] leading to the conclusion that the lesions developed as a result of the degeneration of specific cortical pathways [28].

4.2 The negative binomial distribution

The negative binomial distribution can be fitted to a variety of clustered patterns and may give a more accurate estimate of the intensity of clustering. The negative binomial is a two-parameter distribution defined by the mean density of individuals (μ) and the binomial exponent 'k'. The value of 'k' is generally between 0.5 and 3.0 and decreases as clustering increases and hence, the reciprocal of 'k' is used as an index of the degree of clustering. The procedure for fitting the negative binomial to data is given by Cox [29]. Essentially, any sample information about the numbers of objects in space can be analysed as long as the mean number of individuals per sample is low and plot size is adjusted to reflect this limitation. Data are grouped as a frequency distribution to show the number of samples (f) containing various numbers of individuals (X). The mean number of individuals per plot is then calculated and 'k' estimated by an iterative procedure. The expected frequencies of samples containing various numbers of individuals can then be calculated and compared with the observed distribution to test whether the negative binomial is an adequate fit to the data. If the data do fit the distribution, then 1/K will estimate the intensity of aggregation.

4.3 Morisita's index of dispersion

Morisita's index of dispersion [30] is an alternative method of determining clustering and has the additional advantage that the index is unaltered if objects have disappeared at random from the original clusters. This may be especially relevant in the analysis of neuronal populations since cell losses

frequently occur as a consequence of disease. Morisita's index of clustering (I_d) (Table 1) is unity for a random distribution, zero for a perfectly uniform distribution and equal to 'n' when individuals are maximally aggregated. The significance of I_d can be tested by a χ^2 test (Table 1).

4.4 Plotless methods of determining spatial pattern

In Holgate's method [31], a number of randomly selected points ('n' at least 50) are superimposed over the area of the section to be sampled. From each point, the distance to the nearest object of interest (d) is measured and the distance to the second nearest object (d_1). The index of aggregation (A_1) (Table 1) is zero for a random distribution, greater than zero for a contagious distribution, and less than zero for a uniform distribution.

In Hopkin's method [32], a number of points are superimposed at random over the section and the distance of each point to the nearest object measured (d). Second, a total of 'n' objects are selected at random and the distance from each to the nearest object of similar type measured (d_1). The index of aggregation A_2 (Table 1) is zero for a random distribution, greater than zero for a contagious distribution, and less than zero for a uniform distribution.

5. Measuring spatial correlation (Table 2)

In many neurodegenerative diseases, there may be more than one type of lesion present in the tissue, e.g., AD is defined by the presence of both extracellular Aβ deposits and intracellular NFT and CJD by the presence of vacuolation ('spongiform change') and PrP deposits. The degree of spatial association between the various lesions and between lesions and normal features of the brain such as blood vessels or neurons may be useful information in elucidating the pathogenesis of the disorder. Several methods are available for testing whether different features are spatially correlated and include methods based on contingency tables and on grids or transects of contiguous plots (Table 2).

Table 2. Formulae and significance tests for studying the association between features in histological sections.

Test	Statistic	Significance test	Data
2 x 2 table	C_7	-	Frequency
2 x 2 table	X^2	χ^2 distribution	Frequency
(k) features	$E(m) = N\Pi(I= 1to\ k)(N - n_i/N)$	Normal (z test)	Frequency
Two features	Covariance	-	Any quantitative measure
Two features	Pearson's correlation' coefficient ('r')	r' distribution	Any quantitative measure
Several Features	'r' matrix, stepwise' multiple regression	r','F' distributions	Any quantitative measure

NA = not available; C_7 = Coefficient of association, χ^2 = chi-square, X^2 = chi-square corrected for continuity, V = Variance, E(m) = Expected number of empty plots, N = total frequency, n_i = number of plots with the 'ith' histological feature, k = number of histological features, r = correlation coefficient, F = Variance ratio

5.1 The coefficient of association (C_7)

A simple method of testing for spatial association between two histological features uses qualitative data arranged in a 2 x 2 contingency table. Plots (N) are located at random within a tissue section and the presence or absence of two histological features (A,B) are recorded within each sample unit. From the

contingency table, a coefficient of association (C_7) can be calculated which varies from +1, when the maximum possible co-occurrence is present, to -1 the minimum possible co-occurrence. Values close to zero indicate that the frequencies of the two features are similar to those that would be expected to occur by chance. The actual calculation of C_7 depends on the relationship between the numerical values in the contingency table and is dependent on first, whether the product of the joint presences and joint absences is greater or less than the product of samples which contain one feature alone and second, on the relative magnitude of the individual frequencies [33]. This method was used to determine the degree of spatial association between SP and NFT in AD [34].

5.2 Chi-square (χ^2) in 2 x 2 contingency tables

An alternative approach to the statistic C_7 is to calculate χ^2 from the frequencies in the contingency table. Chi-square is a test of the null hypothesis that the two histological features are distributed independently. Since the χ^2 distribution is continuous and is being used in this case to approximate to a discrete distribution, it is necessary to make a 'correction for continuity' [35] (Table 2) and this statistic is usually given the symbol X^2.

5.3 Correlation between more than two histological features

It may be necessary to explore the joint occurrences of more than two histological features, e.g., whether the different morphological types of Aβ deposit (diffuse, primitive, classic, and compact) occur together more often than chance would suggest [4,29]. With more than two features, the contingency tables become complex, but it is still possible to determine whether the features as a whole are positively associated (Table 2). A group of 'k' histological features will be positively associated as a whole if an unexpectedly large number of plots contain representatives of all of them. Hence, it is possible to test the difference between the observed and expected frequencies for the class defined by the joint occurrences of all the features [35]. Similarly, it would be possible to test the number of units containing no individuals of any of the histological features or 'empty' plots (M).

5.4 Correlation based on contiguous plots

If the plots are arranged in a grid or as a transect, it is possible to study the effect of plot size and distance on the nature of the correlation between two features and therefore, to establish the scale at which the correlation is most evident. For example, a significant correlation between Aβ deposits and blood vessels in AD using small plots approximating to the size of individual plaques, would suggest a close relationship between the two features. By contrast, if correlation was only present in much larger plots, then it could be fortuitous, resulting from the abundance and widespread distribution of the deposits and blood vessels in the AD brain.

This aspect of spatial pattern analysis can be studied by the use of a correlation coefficient. A quantitative measure such as density, coverage, or load is obtained for two histological features in a series of plots arranged contiguously. Quantitative measurements in adjacent fields are added together successively to provide data for larger field sizes, e.g., two unit blocks, four unit blocks etc., up to a field size limited by the length of the transect. Pearson's correlation coefficient ('r') or a non-parametric correlation coefficient can be used to determine correlation at each field size. An example of the use of this method in AD to study the spatial correlation between the diffuse-type of Aβ deposits and associated cells is shown in Fig 4. There is a spatial correlation between the diffuse deposits and neuronal cell bodies at field sizes 200, 400, and 800μm indicating a close spatial relationship at distances less than 800μm. By contrast, glial cells are only weakly correlated with the diffuse deposits at field size 3200μm. These data suggest that the development of the diffuse deposits in AD is specifically related to neuronal cell bodies rather than to glial cells, the correlation with glial cells at the larger field size reflecting the

general abundance of deposits and glial cells in the section. The spatial correlation between several histological features can be studied using a correlation analysis and by stepwise multiple regression [33].

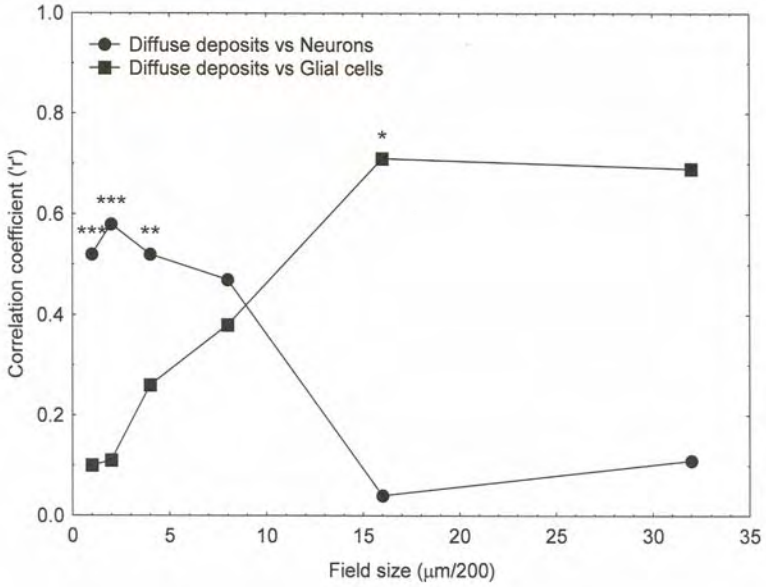

Fig 4. Spatial correlations between the diffuse β-amyloid (Aβ) deposits and associated cells in the temporal cortex of a case of Alzheimer's disease (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

6. Discussion and conclusions

Quantitative analysis has become an important aspect in the pathological diagnosis of neurodegenerative disease [4] and in studies of disease pathogenesis [27]. Hence, application of appropriate sampling strategies, quantitative measures of abundance, and data analysis methods have become an important part of neuropathological methodology.

A quantitative measurement needs to be relevant to the specific objectives of the study. Frequency measurements or semi-quantitative scores often provide a quick and easy method of indicating the abundance of an object in the tissue but lack precision and the former are highly dependent on the size and shape of the plots. They are therefore useful in preliminary quantitative surveys of different pathological cases and may provide sufficiently accurate data for a large-scale study of heterogeneity within a particular disorder using a method such as Principal component analysis (PCA) [23]. Density measurements, by contrast, provide the most reliable measure of lesion abundance, and are essential for studying spatial patterns and correlations between different types of histological feature, but are time-consuming to obtain for large numbers of patients. In some circumstances, individual objects cannot be identified and coverage or load may be a more appropriate measure of abundance than density.

A number of different methods are available for measuring the spatial pattern of a histological feature [5,17]. If the objective is simply to determine whether a feature is distributed at random in a tissue, then one of the methods based on the V/M ratio could be used. A major disadvantage of the simpler methods is that spatial pattern detected is markedly affected by the size and shape of the plots. In addition, brain lesions often exhibit a complex spatial pattern in brain tissue [27] with small clusters of lesions regularly distributed parallel to the tissue boundary and further aggregated into larger-scale clusters. In these cases, analyses based on grids or transects are essential in providing information on the different scales of clustering present. An alternative strategy is to use a plotless method of sampling that involves determining the distance between an object and its nearest neighbour of the same type. A problem in applying these methods is that an object nearest to a random point cannot be taken as a 'randomly chosen' member of the population as this would result in a biased sample [35]. Hence, a random

individual would have to be selected from the population as a whole. This would require a complete census of the population and would be a very difficult task if the feature was especially numerous.

Methods determining the degree of correlation between histological features fall into two distinct categories, viz., those based on contingency tables in which the presence and absence of lesions are analyzed and more quantitative methods which use transects or grids of contiguous plots. Methods based on contingency tables are particularly useful in preliminary studies to determine whether there is a positive or negative correlation between lesions that should then be investigated in more detail. Of the statistics that can be used on contingency tables, C_7 is influenced by the ratios of the frequencies in the different cells of the table. The χ^2 test is less sensitive to this problem and hence, should always be calculated along with C_7. The limitation of contingency table methods is that they rely on recording the joint presences and absences of features in defined sample plots. The correlation between two histological features in AD, however, may be more complex. For example, a large blood vessel might influence the pathogenesis of Aβ deposits for some distance around the blood vessel, not just in the plots that actually contain the vessel profile [21]. In these circumstances, quantitative data collected in contiguous plots at different distances from the blood vessel provide a much more accurate assessment of association. Association can be measured in this context either by analysis of covariance or by Pearson's correlation coefficient [5]. The disadvantage of the former is that it is difficult to make a test of significance whereas in the latter, the test assumes that the data are a sample from a bivariate normal distribution.

References

[1] Bruce CV, Clinton J, Gentleman SM, Roberts GW, Royston MC, Neuropathololology and Applied Neurobiology **18**,125 (1992).
[2] MacDonald ST , Sutherland K, Ironside JW, Neurodegeneration **5**, 87 (1996)
[3] Syed AB, Armstrong RA, Smith CUM, Alzheimer Reports **3**, 19 (2000).
[4] Armstrong RA, Histopathology **42**, 521 (2003a).
[5] Armstrong RA, Neurodegeneration **2**, 73 (1993a).
[6] Brower JE, Zar JH, von Ende CN, Field and Laboratory Methods for General Ecology. Wm C Brown, Dubuque, IA, USA, (1990).
[7] Cummings BJ, Cotman CW, Lancet **346**, 1524 (1995).
[8] Armstrong RA, Lantos PL, Cairns NJ, Pathophysiology **8**, 99 (2001a).
[9] DeLacoste M, White CL. Neurobiology of Aging **14**, 1 (1993).
[10] Duyckaerts C, Hauw JJ, Bastenaire F, Piette F, Poulain C, Raisard V, Javoy- Agid F, Berthaux P, Acta Neuropathologica **70**, 249 (1986).
[11] Hof PR, Morrison JH, Journal of Comparative Neurology **301**, 55 (1990).
[12] Armstrong RA, Alzheimer Reports **3**, 133 (2000).
[13] Armstrong RA, Cairns NJ, Lantos PL, Neuroscience Letters **242**, 81 (1998b).
[14] Pearson RCA, Esiri MM, Hiorns RW, Wilcock GK, Powell TPS, Proceedings of the Natational Academy of Science, USA **82**, 4531 (1985).
[15] Delaere P, Duyckaerts C, He Y, Piette F, Hauw JJ, Acta Neuropathologica **81**, 328 (1991).
[16] Schultz-Schaeffer WJ, Giese A, Windl O, Kretschmar HA, Clinical Neuropathology **15**, 353 (1996).
[17] Armstrong RA, Journal of Neuroscience Methods **73**, 141 (1997).
[18] Sutherland K, Ironside JW, Analys is of Quantitative Cytology and Histology **16**, 430 (1994).
[19] Sutherland K, MacDonald ST, Ironside JW, Journal of Neuroscience Methods **64**, 123 (1996).
[20] Cairns NJ, Chadwick A, Luthert PJ, Lantos PL, Neurosci Lett **129**, 115 (1991).
[21] Armstrong RA, Current Neurovascular Research **3**, 289 (2006a).
[22] Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L, Neurology **41**, 479 (1991).
[23] Armstrong RA, Nochlin D, Bird TD, Neuropathology **20**, 31 (2000).
[24] Armstrong RA, Neuroscience Letters **160**, 57 (1993b).
[25] Armstrong RA, Cairns NJ, Lantos PL, Neuroscience Letters **224**, 41 (1997b).
[26] Armstrong RA, Lantos PL, Cairns NJ, Movement Disorders **19**, 109 (2003).
[27] Armstrong RA, Lantos PL, Cairns NJ, Neuropathology **21**, 1 (2001b).

[28] Armstrong RA, Journal of Microscopy **221**, 153 (2006b).
[29] Cox GW, Laboratory Manual of General Ecology. Wm. M. Brown, Dubuque, IA,USA, (1990).
[30] Morisita M, Memoirs of the Faculty of Science Kyushu University Series E (Biology) **2:** 215 (1959).
[31] Holgate P, Jornal of Ecology **53**, 261 (1965).
[32] Hopkins B, Annals of Botany **18**, 213 (1954).
[33] Armstrong RA, Neuropathology **23**, 245 (2003b).
[34] Armstrong RA, Folia Neuropathologica **43**, 133 (2005).
[35] Pielou EC, An Introduction to Mathematical Ecology, John Wiley, New York, (1967).