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QUANTITATIVE HISTOPATHOLOGY
DISCOURSE

QUANTITATIVE NUCLEOLOGY: THE QUANTITATIVE ASPECTS OF THE STUDY OF NUCLEI

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ABSTRACT

The quantitation of nuclear features has proved attractive to those wishing to find cellular variables of diagnostic and prognostic value. The emerging disciplines of morphometry, stereology, static and flow cytometry, and three dimensional reconstruction, enhanced and spurred on by new computer systems and statistical methods, are being pressed into service in many laboratories. As yet only a few simple prognostic indices, applicable to specific cancers, for example of the eye, breast and bladder, have reached routine clinical practice, but the many nuclear variables under examination are reviewed and put into context.

Keywords: cancer, morphometry, nuclei, nucleoli, prognosis, stereology.

INTRODUCTION

It has long been observed in histological and cytological preparations that the nuclei of tumour cells vary more in size, shape and staining than, and differ in these respects from, the nuclei of the relevant normal cells. Karyometry or nucleometry is directed towards quantifying these differences and is now a growth industry. Indeed, of the 80 papers published in the first three volumes of Analytical Cellular Pathology (1989-1991), 50 include nuclear quantitation.

The differences detected by nucleometry are studied either for what they reveal about the biology of the tumour, in terms of cell proliferation, differentiation, invasive or metastatic potential, or to correlate them, either individually or in some multivariate manner (Montironi 1991), with some measure of the natural history of the tumour. From these correlations prognostic indices applicable to the tumours of individual patients have been developed and used to devise appropriate treatment protocols for clinical trials. Thus nuclear morphometry is being widely used for tumour research and is gradually entering into oncological practice.

Quantitative nucleology suffers, however, as a diagnostic tool in that tumour entities are usually established on qualitative histological criteria so that much effort has gone into identifying quantitative differences between entities, the "gold standards" of which are themselves subjectively defined. This is often extended to grading of tumours whereby subjectively determined grades of tumour or of dysplasia are then assessed for quantitative differences. The relationships between qualitative and quantitative diagnostic criteria and grading criteria, and thence prognosis, need further rigorous thought by those involved in all aspects of oncology.

TECHNIQUES

The techniques at present available for quantitative nucleology fall into five groups - nuclear morphometry, nuclear stereology, static DNA nucleometry (not further considered in this paper), flow DNA nucleometry (not further considered in this paper), and three dimensional nuclear reconstruction.

Common to each are the problems of obtaining, sampling, and processing the tissues containing the nuclei, selecting the nuclei to be studied, and then making and analysing the nuclear measurements so that the results are both reproducible and meaningful in relation to some prognostic variable. There are many pitfalls, and the translation of the fruits of quantitative nucleology into clinical practice is definitely inhibited by the doubts of readers (pathologists and clinicians) about whether one or other procedure for predicting tumour behaviour is reliable. It is also inhibited by the reluctance of pathologists to incorporate quantitation into their routine surgical pathology work unless they can be sure that the results are really necessary. This is because quantitation consumes much more time than qualitative slide reading, at the present level of education takes a big initial effort for the pathologist to understand properly, and may need expensive equipment and staff. Thus probably the only universally accepted quantitative variables in routine surgical pathology are the highly questionable counting of mitoses per "high power field" in some sarcomas and carcinomas and Breslow's thickness of malignant melanomas, although semi-quantitative grading of tumours has also entered practice. Quantitation is also becoming routine in the assessment of muscle biopsies and metabolic bone disease.

NUCLEAR MOPHOMETRY

Much ingenuity has gone into devising nuclear variables to measure in tissue sections. These range from simple counts of total nuclear number or the proportion of nuclei in mitosis or showing some feature such as a proliferation marker, to syntactic structure analysis (minimum spanning tree) (Table 1).

Table 1. Nuclear Variables

a. item counts eg mitoses b. nuclei - area - diameter - perimeter - longest axis - shortest axis - Feret X - Feret Y - shape factors - volume c. nucleoli - frequency - area - diameter - perimeter - longest axis - shortest axis - shape factors - nucleolar position - volume - position (nucleolar organising regions: see below)	d. texture e. syntactic structure analysis - minimum spanning tree f. markers associated with nuclear activity - thymidine labelling - bromodeoxyuridine (BrdU) labelling - Ki67 - PCNA - nucleolar organising regions

Mitotic counts

Fleege et al (1991) have laid down criteria for reproducible mitotic counts. These include the criteria for the recognition of a mitosis, for the selection of the area in which the mitoses are to be counted, and for the microscopy and counting procedure. These authors use the number of mitotic figures in 10 consecutive fields at x400 magnification with a numerical aperture of 0.75 and a field width of 450μm for their mitotic activity index (MAI) with high reproducibility. The MAI thus relates the number of mitoses to areas of tissue in the section; absolute or total counts of items unrelated to anything are rarely of value. The MAI correlates well with survival, particularly in breast carcinomas, especially when combined with node positivity and tumour size in a multivariate prognostic index (Baak and van Diest 1991). Collan and his colleagues, however, have studied the reproducibility of mitosis counts (Montironi et al 1988) and related mitotic index specifically to tumour tissue by dividing the mitotic index by the volume fraction (see below) of the neoplastic epithelium in the same 10 fields (Haapasalo et al 1989) and found the resulting "volume corrected mitotic index" to be the best biological prognostic indicator in ovarian cancers (Haapasalo et al 1989) and efficient for grading in bladder carcinomas (Lipponen et al 1990).

Nuclei

Without computerised morphometric systems straight line nuclear measurements can be made with eyepiece and stage micrometers; curvilinear measurements with a planimeter on photomicrographs. The computerised systems recently taken into general use have, however, greatly increased the ease, speed and accuracy with which the measurements in Table 1 can be made.

These systems depend on a human being drawing the outlines of individual nuclei one by one on a digitising tablet. This generates x,y coordinates which are translated by the computer into the measurements required and, by manipulation of the measurements, into derived variables for individual nuclei, such as shape factors. Statistical comparisons between groups of nuclear measurements and statistics derived from them are easily made, and can usually be shown graphically on the screen as bar charts, scattergrams, regression lines and so on. An enormous amount of information can be generated from the x,y coordinates of two groups of normal and tumour nuclei. Many of the 50 papers referred to above are concerned with this type of information.

In the interactive type of computerised nuclear morphometry outlined above the human operator can approach the microscopic image either with a drawing tube attached to the microscope, or through the projected image or with a camera interface video overlay. The latter shows the microscope image on the monitor (preferably high resolution and flicker free) with the digitised drawing superimposed on it, often together, in some systems, with the menu for the measurements available.

It has been suggested that it would be even better if the measurements could be made automatically without the interaction of a human operator. Much work has been done to achieve this, for example by Sowter et al (1990). However, this approach is not routinely available. The difficulty lies in programming the computer to recognise the items to be measured, many of which are usually not clearly delineated from other items in the slide or from each other. Sowter was able to overcome some of these problems in relation to the nuclei of bladder neoplasms.

The problems associated with obtaining reproducible interactive nuclear morphometric data have gradually been identified and addressed by Fleege and his colleagues (1991). The advice in Table 2 is based on their findings. Unfortunately in nuclear morphometry

Table 2. Guidelines for morphometry of nuclei

1.	Do not freeze the tissue or allow it to dry.
2.	Fix representative slices of tumour in buffered 4% formaldehyde at pH 5-9 for not less than 6 hours and not more than 72 hours.
3.	Regularly check formaldehyde pH.
4.	Take at least 10 blocks from any tumour or one section per cm diameter or 100g in large tumours.
5.	For digitising on the digitising tablet nuclei should exceed 15mm. Use cursor in preference to pen.
6.	For sampling prefer the raster method for histology, the zone method for cytological preparations.
7.	Start with adequate training and maintain quality control.
8.	For more detailed explanation and advice refer to Fleege et al 1991.

papers one is seldom told how these threats to reproducibility have been addressed if at all.

Nucleoli

Nucleoli have not received the same attention as whole nuclei. Nevertheless standard deviation of nucleolar area is a reliable and reproducible predictor of mortality following enucleation of the eye for intraocular melanoma (Gamel et al 1982; Huntington et al 1989). Nucleoli in breast cancer are of possible prognostic value (van Diest et al 1990); the number of nucleoli per 100 nuclei proving to be the best single prognostic variable. In addition to percentage nucleolated cells Montironi et al (1990) have noted percentage nucleolar margination as the best discriminatory feature in distinguishing thyroid adenomas from carcinomas. (Nucleolar organising regions will be considered below).

Shape factors

Tumour nuclei are believed to be more "pleomorphic", ie. more variable in shape, size and staining, than normal nuclei. Manipulations of morphometric data have therefore been attempted to evaluate variation in shape. For example Form PE ($4 \times \pi \times$ area/perimeter^2) = 1 for a circle and <1 for an ellipse or irregular structures; Form AR ($1/4 \times \pi \times$ (longest axis) x (shortest axis) = 1 for circle or ellipse and <1 for irregular structures; Form P2A (perimeter^2/area x 4π) = 1 for a circle; bending energy (Young et al 1974); perimeter indentation measurements; standard deviation of the distance of the nuclear boundary from the centre of gravity of the nucleus (Jagoe et al 1984) have been devised. These and others were tested on 20 drawn shapes by Sowter (1990) who found that many were size dependent and only P2A was shape specific, but could give similar values for dissimilar shapes. Pesce Delfino's group, also concerned that the shape descriptors currently in use were not sensitive, have applied mathematical analytical procedures to the nuclei and nucleoli of lymphoid cells to produce size-independent nondimensional variables using a Shape Analytical Morphometry (SAM) workstation. Multivariate analysis of the results has given good discrimination between centroblastic and immunoblastic lymphomas (Ricco et al 1989).

Texture analysis

Texture analysis is made from the measurements derived from the grey values contained in pixels over a nucleus and the variations between them. Pressman (1976) has distinguished between cervical cells by "co-occurrence texture features", for the use of which, however, Sowter's computer memory was too limited. Another approach is to look at grey level run length matrices (Galloway 1975). Sowter (1990), however, found that, even with very careful control of staining and illumination, variations in section thickness gave gross variations in mean grey value.

Syntactic structure analysis

This is the approach of graph theory to the spatial relationships between nuclei, whereby the centres of gravity ("vertices") of the nuclei in a field are identified and connected according to specified rules. A tree graph containing all vertices but only those connections having minimum distance is called a "minimum spanning tree". This approach has been taken to Feulgen stained sections of lung cancers using image processing (Kayser et al 1990), and more recently to sections of breast carcinomas stained with haematoxylin and eosin (for example van Diest 1990), in which the centres of gravity of all the malignant nuclei in a field shown on the monitor of a digitising interactive video overlay system were marked and the minimum spanning tree applied. Many variables can be calculated with good reproducibility and appear to be related to tumour differentiation. The value of this approach must await much further use and evaluation.

Proliferation markers

The sum of tumour cell proliferation and tumour cell death constitutes tumour growth. Attempts have therefore been made to see whether tumour cell proliferation is related to tumour behaviour or prognosis. Once again attention has focussed on the tumour cell nuclei, in particular to identify the proportion of cells that are actively cycling. Actively cycling cells, ie those heading for mitotic division, are regarded as proliferating. Because a fraction of these cells (the fraction in S phase) synthesise DNA they may be labelled by tritiated thymidine which is detected in the nuclei by autoradiography or by bromodeoxyuridine (BrdU) which is detected in the nuclei by monocloncal antibodies (Gratzner 1982). Alternatively nuclear antigens associated with cycling cells, such as Ki67 (Gerdes et al 1983) or proliferating cell nuclear antigen (Miyachi et al 1978), may also be identified by monoclonal antibodies. These techniques have many theoretical and practical problems but the morphometry consists simply of identifying the marked nuclei and expressing them as a proportion of the total nuclei as an index. Another approach is that of counting nucleolar organising regions after silver staining (AgNORs) (Howell and Black 1980). The number of AgNORs per cell have been shown to increase with nuclear and cellular activity.
These techniques have been applied to the study of the nuclei of many tumours. We have applied the AgNOR and Ki67 techniques particularly to a series of lung tumours but found no correlation in the results obtained by the two techniques (Soomro and Whimster 1990).

NUCLEAR STEREOLOGY

Whereas morphometry is a set of methods whereby measurements are made of all or a sample of items or elements in a tissue, stereology is a set of methods whereby the tissue is sampled and the measurements are transformed so that they are relevant in the 3-dimensional space and not only in the 2-dimensional space of the microscope image.

If the total volume of the organ or tissue is known, the absolute number, area or volume of the element of interest may be estimated from the proportions obtained above. The total volume can be readily estimated by the principle of Cavalieri (1598-1647) whereby the volume of the sliced organ or tissue is obtained by multiplying the sum of the areas (obtainable by point counting, see below) of the slices by the interval between the slices. The earliest stereological principles were developed by geologists to estimate the volume fraction of diffusely distributed components of rock from the area fraction of the same component on a cut surface (Delesse 1847). The microscopic eyepiece graticule was a basic tool, but the interactive computer systems mentioned above can also include a graticule overlay. The proportion of the area of a microscope field that is occupied, for example, by malignant epithelium may be obtained by point counting, when the number of points falling on the epithelium is taken as a proportion of the total number of points on the image. The area of epithelium is in the same proportion as the points, so, if the total area is known, the area of epithelium per unit area of tissue may be calculated; similarly the volume is in the same proportion as the points, so, if the total volume of tissue is known, the volume of epithelium per unit volume of tissue may also be calculated.

The area fraction is used together with mitosis counts to give the volume-corrected mitotic index mentioned above (Haapasalo et al 1989). The latter index is in linear relation to the number of mitotic figures per volume of epithelium (Collan 1991). Thus morphometric and stereologic techniques may be usefully combined.

Point counting is very efficient. The number of points that must be counted for a given percentage standard error is easily calculated (Turner and Whimster 1981). The density of the graticule test system should be such that each element is seldom hit by more than two neighbouring points. If a graticule consisting of a series of lines is superimposed on the field, the number of intersections of the epithelium per unit line length can be used to estimate the surface area of the epithelium.

A new volume variable, the volume-weighted mean volume, has recently been put forward for arbitrarily shaped particles, such as nuclei (Gundersen and Jensen 1985). Volume weighted refers to sampling by points randomly positioned on a section - such points hit a nucleus with a probability which is directly related to the volume of the nucleus. A linear graticule, with marked points on the lines, is superimposed on a field. Where a point overlies a nucleus, the corresponding segment is classified from one to ten by a ruler which divided into 10 equal lengths numbered one to ten. The length of the ruler should not, of course, be smaller than the largest nucleus. For a ruler length, l mm, the length of the first class is $\sqrt[3]{1 \times (l\ mm)^3/10}$, the second class $\sqrt[3]{2 \times (l\ mm)^3/10}$, etc. After the required number of nuclei (say 100) have been classified, and knowing the final magnification of the image (often an EM photograph) and the length of the ruler, the mean intercept length^3 (l_o^3) can be calculated as magnification factor x [(n x class 1 mm) + (n x class 2 mm)....+ (n x class 10 mm)]/ 100. $l_o^3 \times \pi/3\ \mu m^3$ gives the volume-weighted mean volume.

Point counting can also be used to represent absolute area instead of area fraction of an element in tissue sections (Lord et al 1978). The Cavalieri principle (see above) can then be applied to sum the absolute areas on serial or interval sections to give absolute volume, as in emphysema in whole lungs (Turner and Whimster 1981). Even with this approach it may be necessary to express volume in relation to some other unit to allow comparisons, for example the variable, volume of bronchial gland per unit surface area of bronchial wall lumen, can be used to compare the amount of bronchial gland between bronchi of different dimensions whereas absolute volume of bronchial gland cannot (Whimster et al 1984). Similar approaches may also be applied to nuclear area or volume measurement.

New and efficient stereological methods have been introduced in recent years, particularly to eliminate bias and to overcome inaccuracies introduced by anisotropy of biological ma-

terial. These are not easy to understand but are comprehensively reviewed by Gunderson et al (1988a,b). A particularly valuable and hitherto unattainable application is that of estimating the number of particles (cells, nuclei) in three dimensional space. The particles are observed in two tissue sections a known distance apart, and the number of particles in one plane but not the other are counted. The two sections are most conveniently observed by focussing through a thick section and measuring the depth with a measuring device (microcator) or with a confocal microscope.

THREE DIMENSIONAL RECONSTRUCTION OF NUCLEI

Another approach to nuclear volume and surface area measurements, as yet little exploited, is offered by computerised three dimensional reconstruction techniques, such as that of Cookson, Holman and Dykes (Whimster and Cookson 1991). These may be applied either to serial EM photographs or to successive computerised images taken through nuclei in tissue sections with a confocal microscope. Having collected the data it is quite easy to see that the whole nucleus has been included in the stack of images by viewing it from the side. Although the techniques are now fairly well worked out, systematic measurement of nuclei by these means has not yet been undertaken.

RELATING NUCLEAR NUCLEOMETRY TO TUMOUR BEHAVIOUR

Measurements of tumour nuclei obviously have to be compared with measurements of normal nuclei and equally obviously have to be related to tumour behaviour if they are to be of clinical value. Tumour behaviour may be assessed in terms of its growth, the development of metastases or the duration of survival of the patient. The identification of any of these outcomes is fraught with obvious difficulties. One would think that death would be an easy end point, requiring only patience. Unfortunately in many countries many patients are lost to follow up. In those tracked down it is often difficult to determine reliably whether the patient was killed by the tumour, died from some other cause but with residual primary or metastatic tumour, or died free of the tumour. One suspects that in many reports the meticulous work that has often gone into making the nuclear measurements is not matched by that of the survival or tumour behaviour data, thus casting a shadow over the validity of the whole work.

However, having got the nucleometry and the time intervals between some starting point, eg first symptom or diagnosis, and death, there are now several ways of relating individual nuclear variables to the duration of survival. Survival probability tables can be constructed (Hill 1959) although it is now more usual to publish the results in graph form, with probability set against time for the variables concerned, as we have done with two groups of Ki67 results in patients with lung cancer (Soomro et al 1991). The statistical significance of the difference between the curves may be assessed by the chi-squared test. Care must be taken over patients lost to follow up.

More recently the Kaplan-Meier method has been widely adopted, in which a drop occurs only when a death occurs (Kaplan and Meier 1958). Many statistical packages include this but a useful public-domain ('shareware') microcomputer program for exploratory univariate statistical analysis of survival data which calculates life table-like information based on Kaplan-Meier's product-limit estimators of the survivorship function S(t) is KMSURV. It includes the Mantel-Cox and Breslow's generalised Wilcoxon tests for comparison of the survival distributions defined by the grouping variable. (KMSURV is available from Epidemiology Monitor, 2560 Whisper Wind Court, Roswell, GA 30076, USA, for a small mailing charge of $5).

Multivariate analysis is needed to assess the value of multiple prognostic variables. Cox's regression models of proportional hazards are often used.

PUBLICATION

Readers have a harder time with papers that include quantitative nucleometry than with biomedical papers generally. Authors assume that their readers, including the referees, will be thoroughly at home with the relevant medicine and pathology, cell biology, computer science, and statistics and with the principles of paper writing in English (Whimster 1977). If, not surprisingly, they are somewhat confused or ignorant in one or other area themselves, the writing is likely to be less than crystal clear. This does the subject a disservice (Whimster 1985)

The first result of lack of thought for the reader is usually that the message of the paper is difficult to find; and then that the reader has great difficulty in deciding whether he can believe the message on the evidence presented to him. The methods section is often inadequate - if it is not clear how the tissue was obtained, fixed, sampled, processed, or stained, how the slides were sampled, how the measurements were made, or what statistical methods were used, or why these procedures were chosen, it is impossible for the reader even to imagine himself repeating the work, or understanding, let alone trusting, what was done.

Secondly the raw results are often not presented in such a way that the reader can see if they are normally distributed, with what outliers, so he cannot obtain an overall "common-sense" impression of the relationships between the groups under comparison. The derivation of the derived results may not be clear. One difficulty is that the results of too many experiments are often presented in the same paper, so that the reader cannot keep track of which experiment is being compared with which. This confusion may spread into the discussion, which may not be set out in the same sequence as the results, so that the reader finds his mind being torn from one comment to another without a logical progression. The clinical and/or scientific relevance of the work may not be made clear. Much also depends on a well written introduction to set the scene for why the work was embarked upon at all. Finally there may be comments and even data in the abstract which are not in the text, although these have different functions and should each be capable of standing alone.

Unfortunately published papers are the currency of biomedical professional life so the forces towards publishing everything quickly, whether trivial or complete, meaningless or inelegant, are strong. Nevertheless observation of the principles of reader-friendliness as outlined above will go some way to making quantitative nucleology easier to understand.

CONCLUSION

Quantitative nucleology is becoming established more clearly as a component of tumour research and oncological practice but those who practice it need to be acquainted with a wide range of disciplines, notably morphometry, stereology, static and flow cytometry, three dimensional reconstruction, cell biology, computer science and statistics, all of which are developing very quickly. It seems incontrovertible today that the key to cancer lies in the nucleus; it remains to be seen whether the key to cancer control lies wholly or even partly in nuclear quantitation.

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