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REVIEW ARTICLE

VIDEO IMAGE ANALYSIS IN PATHOLOGY

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INTRODUCTION

The need to improve the techniques used by pathologists for the assessment of features in microscopic images, both for diagnostic and research purposes, has been increasingly recognised over the past three decades. Conventional observational microscopy is an 'art' which requires extensive training and experience before the maximum useful information within a stained tissue section can be gained. In the diagnostic arena this usually results in an opinion or diagnosis which represents no more than an intuitive probability statement, albeit in many circumstances one of relatively high value, particularly when the diagnosis relies on the presence or absence of a single clearly defined feature. The ability to produce statements of this quality has increased in the recent past by the use of such techniques as electron microscopy, immunohistology and *in situ* hybridisation. However in most image analysis situations we are required to make an estimate of how often a feature is present in an image or how much there is of a specifically identified product. Here the 'raw' unaided observational methods have been found wanting. Attempts to quantify such information are subject to both random and systematic errors and more importantly, conscious or unconscious observer bias. While observational stereomorphometry (Weibel, 1980; Baak and Oort, 1983; Baak, 1990), involving various structured statistical probing devices, e.g., calibrated eye-piece grids, adds new power to the conventional microscopy and overcomes some of the elements of its subjectivity, it remains a laborious process, difficult to apply in an efficient and adequately reproducible way to any but small scale studies. This is also true even of simple morphometry applied to two dimensional images rather than the more rigorous stereological techniques of Weibel (1980) and others (Bartels *et al.*, 1988; Weid *et al.*, 1989).

Video image analysis (VIA) or computer aided image analysis uses computerised digital image processing techniques to extract numerical information from a visual image, conferring accuracy and automation to the measurement of the microscopic images. Since it is accurate and above all reproducible, less variations can be expected between individual samples in a study and between the observers examining the same samples, provided that rigorous quality control measures are adopted. These features, plus the automatic recording of measurements which can be further processed using standard statistical software, give these systems the advantages of speed and simplicity.

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In this review we will address the general principles which should be followed to achieve high quality results and will draw on our own experience in the use of VIA measurements of features in histological, histochemical and immunopathological studies. We will discuss the use of microcomputer (PC) based systems, monochrome and colour, rather than those based on larger workstations or 'mainframes', in that they are less expensive and relatively portable benchtop systems. There are now many systems of varying levels of sophistication and ease of use on the market, e.g., Video-pro™, Leica, and 'Cue'™, Olympus.

INSTRUMENTATION AND FUNCTIONAL FEATURES OF VIA TECHNOLOGY

Instrumentation

The basic VIA system comprises a standard light microscope (fluorescence or confocal microscopes for special uses), a charge-coupled device (CCD) video camera installed at the tri-nocular head of the microscope, a microcomputer with a digitiser card installed, a mouse or other pointing devices, and a display monitor (Fig. 1). The technical attributes and the software engineering principles of VIA have been reviewed by Jarvis (1992).

Functional Features

The typical VIA system provides user-friendly, simple and fast image analysis with the convenience of a PC. The microscopic image from a particular field is transmitted by a CCD camera onto the display monitor screen as a live video image composed of between 90,000 and 500,000 pixels (the smallest individual components of a video image), the number dependent on the resolution of the cameras, digitiser cards or monitors used. Whichever of these components gives the least pixel resolution will determine the overall resolution achievable in a system. Once an image is chosen for measurement it is captured into memory via the digitiser board and then available on the video screen for subsequent processing using automated or interactive techniques. Various attributes of the stained sections can be examined, e.g., by geometric, densitometric or colorimetric measurements, and the quality of the information improved through thresholding or colour selection, interactive editing, and image processing. The data derived can be analysed by the in-built data analysis programs or transferred into one of a wide variety of PC based statistical and graphical packages, e.g., Statgraphics, Minitab, Lotus, Excelgraph. The operation of the VIA system can be automated to a degree through the user-created macro programs and the addition of a motorised stage.

Colour video cameras are now available with high sensitivity and resolution based on RGB or S-Video standards. The images presented bear a very close resemblance to those viewed in the microscope and features can be deduced on the basis of precise colour identification as well as density, shape and contrast. This broadens the range of editing functions and allows for the precise identification of image components we intend to measure. The colour system is of great advantage for double immunohistochemical staining as different colour products can be differentiated and measured separately even if they overlap.

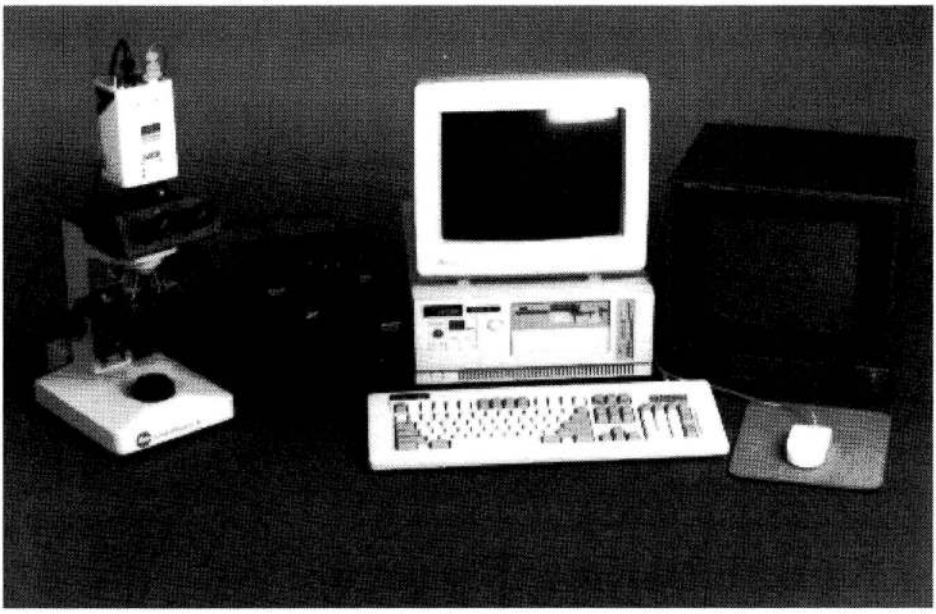

Figure 1. A typical VIA system, comprising microscope with video camera, computer with installed digitiser card and VIA software, plus video monitors. One monitor is used to view the usual computer output for the selection of appropriate programs and sub-routines, the other to display and manipulate the images. In some systems a single monitor suffices. Also shown is a "mouse", used here as a pointer and screen image editing device in addition to the usual computer functions and a video-photographic unit to capture screen images onto film.

The confocal laser scanning microscope (CLASM) in some ways fills the gap between conventional light microscopy and electron microscopy. It allows a higher resolution than a standard light microscopy, using light at a low wavelength (UV or low wavelength blue) to activate a fluorescent dye. At the present time the power of confocal microscopy has been used to increase detection rates for features at the limits of detection in two dimensional images. The real value and power of the confocal however lies in the ability to resolve in three dimensions: XYZ resolution is extremely fine, around 0.2μ. It is a very powerful, if expensive, tool adding functions not otherwise available in routine or fluorescence microscopy.

The earlier confocal instruments have good image detection and enhancement features but do not yet perform at the highest levels as image analysers. However the detector scanner outputs can be handled in a similar fashion to other video output and fed into standard image enhancement and analysis devices. The confocal images of subcellular organelles of cells stained histochemically on sections prepared for light microscopy (paraffin, frozen sections and cultured living cells) were significantly improved by processing them with VIA (Itoh *et al.*, 1992).

Thresholding of Features

In the simplest monochrome VIA systems there are 63 grey levels for the scale of optical densities (OD) with 0 representing the darkest, and 63 the lightest OD. The system

is programmed to recognise and detect features between thresholds preset by the operator to distinguish darker features from their lighter surroundings. There are in practice many instances when strongly and weakly positive staining signals of possible functional or diagnostic significance are present. Such distinctions based upon subjective judgement, however, are prone to significant variations in interpretation which can effect the ultimate diagnostic decision. In a VIA system, a standardised and reproducible distinction between the two different degrees of staining can be achieved by setting two intensity thresholds.

The use of VIA overcomes the inherent 'drift' which inevitably occurs in the observer's internal reference standard in straight visual observations. It has been known for some time that estimates of size for individual features in an image are not constant from field to field (Ooms *et al.*, 1983) and that even greater errors occur in estimates of staining intensity within a single set of observations by one observer as well as between observers. The VIA system also overcomes the problem of errors due to fatigue which occurs during prolonged measurement periods, even for the best trained observers (Dunnill, 1968). The VIA methods enable the establishment of a fixed internal standard which is uniform for all measurement in a study and errors as a result of observer fatigue do not occur.

Editing

Feature thresholding is by no means a complete answer to the problem of isolating particular features for measurement. Features can not always be defined accurately by image intensity alone. Variations in intensity within a feature can be large and overlap with other components of an image under view, with the possibility that some unwanted parts are detected or parts of the features which should be measured are not detected. These deficiencies can be overcome by editing. Various interactive image editing functions are available, such as the ability to erase an unwanted artefact or to measure features within a defined part of a field.

Measurements

After thresholding and editing, the image is ready for either field or feature measurements. In field measurement mode the defined components of an image are measured as a whole regardless of the characteristics of individual features while in feature measurement mode individual features within an image are measured and the information stored for each individual item. For most purposes field measurement is all that is required. The parameters of interest usually measured are the optical density of stain, as well as geometric features: area, the distances between similar features, perimeter and the derivative shape (Fig. 2).

It may also be desirable to calibrate the relative measurements expressed in pixel units into SI units of length, e.g., micrometers, in order to determine the real dimension of a feature. This can be accomplished by the calibration of the pixel units using a stage micrometer in the same way as the familiar calibration of an eye-piece grid.

Image Processing

The VIA systems in common use can perform other functions apart from the basic measurements. Image enhancement techniques can be used to enhance a faintly stained feature, barely recognisable by the eye, to an image of strong contrast by amplifying a

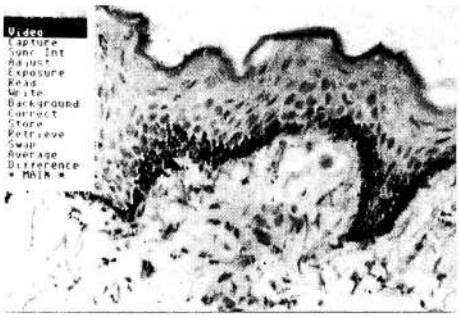

Fig. 2A

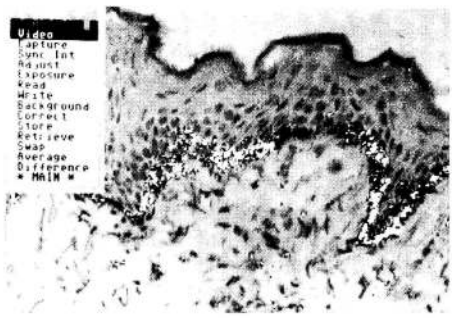

Fig. 2B

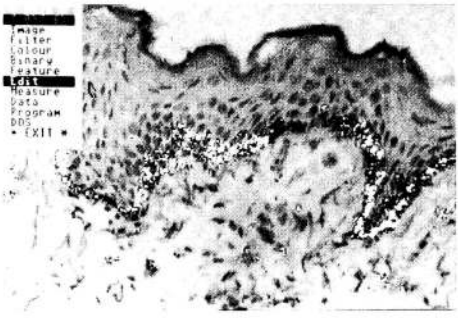

Fig. 2C

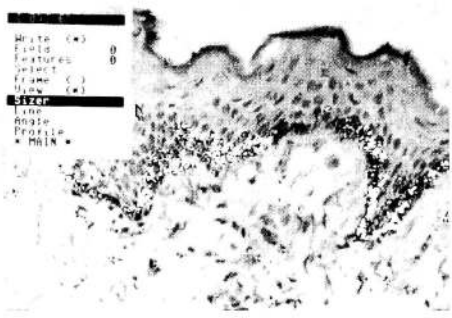

Fig. 2D

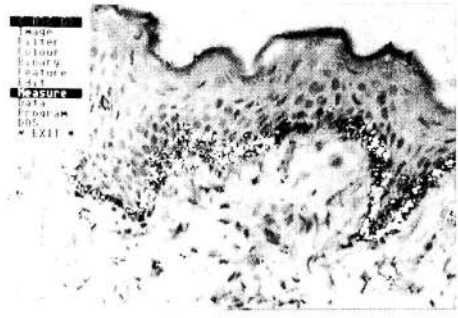

Fig. 2E

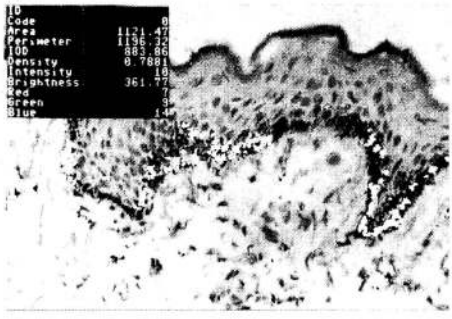

Fig. 2F

Fig. 2. Image capture and manipulation in a specimen of squamous epithelium. 2A: shows the active video image presented on screen. 2B: shows the digitised image with the features to be analysed identified by the white binary overlay. 2C: shows the overlay of the image selected on the basis of colour, and 2D: further selected on the basis of feature size. 2E: shows the area to be measured. At this stage it is usual to 'toggle' between the video and selected images to check on the features to be measured. 2 F: shows the results of analysis of the image for a typical series of parameters in diagnostic practice. Data from a number of fields can be stored in a file and analysed using statistical methods.

small range of grey levels to the full range, using the software feature known as *histogram equalisation*. Thus faintly stained cells can readily be examined, and otherwise unrecognisable features discerned and measured. Another image enhancement function is

inversion where a positive image is inverted into a negative one and vice versa to increase the visual contrast. In monochrome systems the finer structures of a feature can be further enhanced and examined by transforming the grey levels into a visually more distinct spectrum of colours (*pseudo-colouring*), with red usually used for the darkest, through yellow, orange, green and blue to the lighter shades. This colour representation of grey levels helps in the distinction of subtle degrees of difference since it is much easier to distinguish between colours than similar grey levels. Image overlay can be further edited by *erosion-dilation* which acting as a sieve eliminates small features or separates partially overlapping features into distinct ones.

REQUIREMENTS FOR SUCCESSFUL VIA MEASUREMENTS

Field Selection

When following strict stereomorphological principles, the fields examined in a particular case should be chosen at random so as to reduce any degree of observer bias (Fig. 3a). This is only possible for structures in which the components are themselves approximately randomly arrayed, e.g., lung or liver or solid anaplastic carcinomas. However, most structures consist of components with a definite relationship one to the other as in gastrointestinal mucosa. Furthermore it is often appropriate to select a particular component within a field for examination. For example it is appropriate to select only tumour areas if the aim of the study is to measure their feature variability such as nuclear ploidy or to select relevant areas if the cell subtypes in a lymphocytic infiltrate within and around a tumour are to be examined. These problems can be overcome in a number of ways, e.g., by using a system of stratified random sampling, i.e. the origin of the measurement stream is determined by random numbers and from that point onwards the specimen is examined in a stratified manner, the same system being used in every case in a particular study (Dunnill, 1968) or by randomising field selection within defined boundaries (Fig. 3b). Useful information about relationships in a tissue can be achieved by the simple device of serial field selection which gives a cross-sectional representation of cell distribution (Fig. 3c).

There is no straightforward answer to the problem of determining the number of fields to be examined in order to achieve a meaningful result in statistical terms but it can be pragmatically solved by using the progressive mean graph (Aherne and Dunnill, 1982):

$$M_p = \frac{\sum O_f}{f}$$

where M_p is the progressive mean, $\sum O_f$ is the sum of the measured features in all fields, and f is the number of fields in which measurements are made.

This manoeuvre allows for the examination of a sufficient number of fields to achieve a 'smooth' flat curve when expressed graphically. By using this empirical method both systematic sampling and observer bias are reduced. A straight line is rarely achieved as there is always some 'noise' within the system, and for practical purposes we usually accept a 5% variation around the mean as a sufficiently smooth curve. There is no need to measure fields beyond the point where a 'flat curve' is established (Fig. 4).

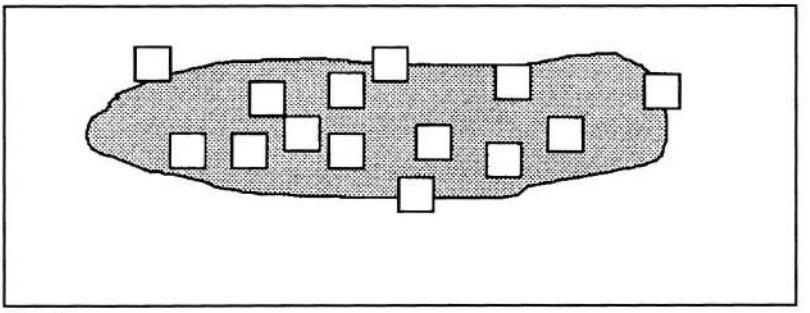

Fig. 3A

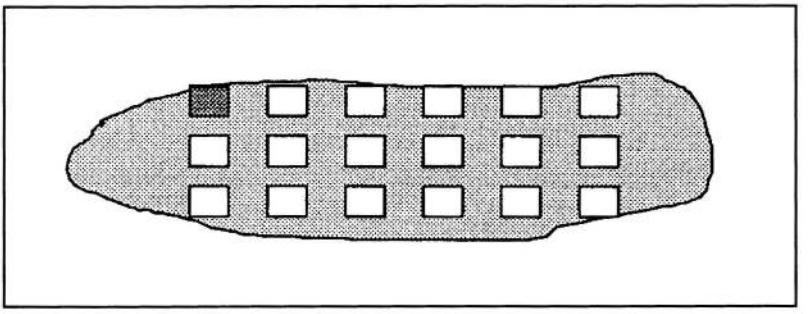

Fig. 3B

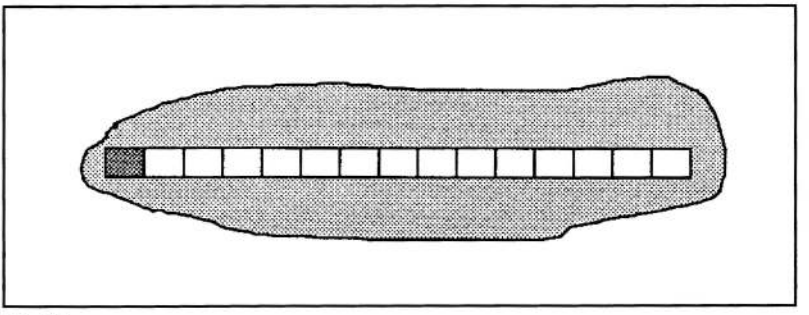

Fig. 3C

Fig. 3. The sampling of fields within a particular section. In (A) the fields are selected in a random manner using a table of random numbers and the microscope stage vernier co-ordinates to determine the particular fields to examine. In (B) a variant is shown. Here the original field (highlighted) is determined randomly and the rest by a predetermined system of stratification. Fig. (C) shows a variation that is useful when the spatial relationships of different features within a field are of interest.

Quality Control

Quality control of measurements is a particular problem in immunohistochemical work. In our laboratory, quality control for a particular antibody at a standardised dilution is achieved by the staining of an appropriate positive control section taken from a block of tissue which has yielded several hundred sections available for use over a long period of time. The standardisation of counting is achieved by measuring a standard sample in

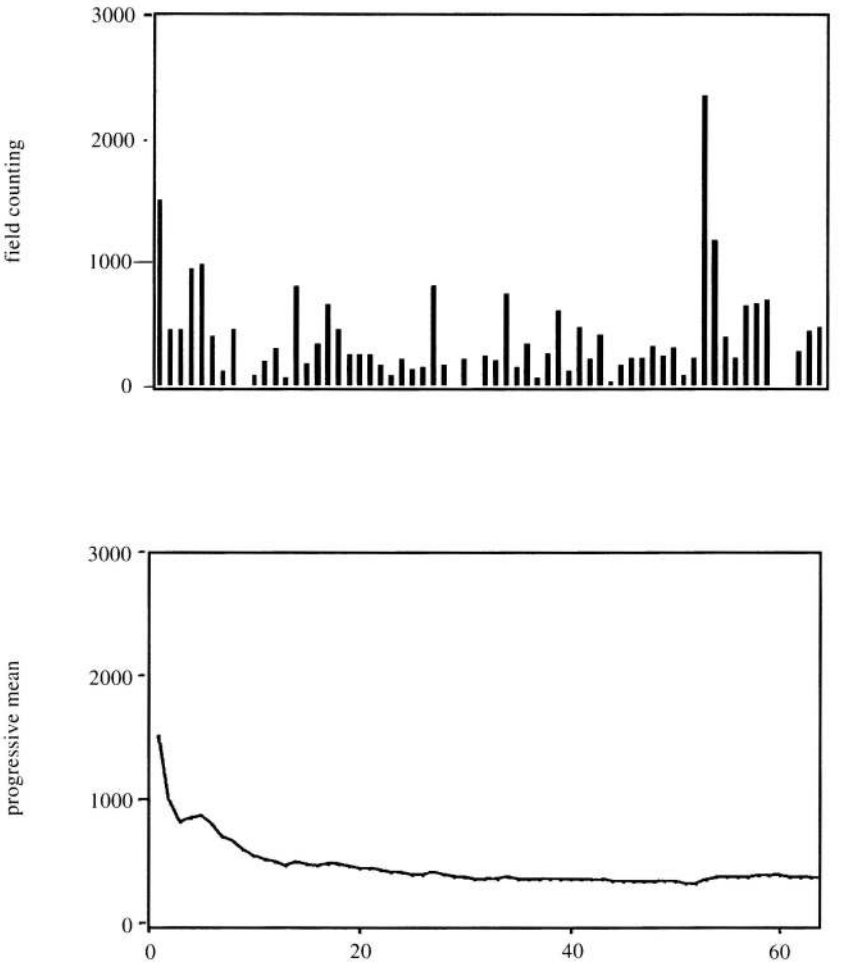

Fig. 4. VIA field counting and progressive mean of a lung section stained with anti-CD3 antibody. The graph of the average staining per field ceases to oscillate after a certain number of fields are counted although individual field counts vary significantly.

every run and reproducibility of measurements by the random selection of previously counted samples which are remeasured in successive measurement batches. Results outside the reference integrated optical density (IOD) range for a particular antibody are rejected. This is in addition to the accepted practice of using positive and negative controls in all immunohistochemical staining runs. The use of a standardised protocol is needed in image identification, enhancement, feature selection and measurement. The light intensity control is critical and always set at the same level. Potential problems such as voltage fluctuation can be reduced either by fitting a voltage control device or by using a photometer.

Careful tissue sampling and preparation must be uniform in all circumstances of a particular study if meaningful comparisons are to be made. Tissues should be removed with care to avoid crushing and either immediately frozen for cryostat work or placed into an appropriate freshly prepared fixative at standardised pH and molality. Fixation should be for the minimum period required for good fixation (determined empirically) and taken straight to the final processing steps. The material can then be stored without concern for quite long periods. We have used material so stored and prepared after intervals of up to 15 years. As the shrinkage of tissue is an important variable in area and shape measurements, both fixation and processing protocols must be standardised and invariable. Control of temperature is important and should be kept within a very narrow range.

Ideally sections should be infinitely thin but this is of course impossible. We cut frozen sections at 4μ and fixed paraffin embedded sections at 2μ. The good attachment of sections to slides is of the utmost importance, especially in immunohistochemical work and *in situ* hybridisation. We always use "superclean" glass slides treated with an adhesive suitable for all the techniques used, e.g., 3-Aminopropyltriethoxy saline, APES, Sigma, and sections rarely became dislodged even after prolonged washing in detergents.

Data Analysis

The in-built data analysis program gives a simple summary calculation of the mean, standard deviation, range, etc., sufficient for an initial evaluation although it is necessary to import the numerical data into one of the standard packages, e.g., Statgraphics, Systat or Minitab, for more careful statistical analysis. The use of these conventional PC data analysis softwares is essential to the successful use of the data generated by VIA allowing one to define meaningful categories by various statistical methods.

Simple descriptive statistics is rarely adequate when analysing a body of morphometric data since the significant measurements and possible interrelationships of features are not always evident. We are usually faced with two distinct situations. In the first the actual groupings are unknown and are to be derived from the data. In this case multivariate analysis is the starting point from which tentative groupings can be developed. The usual approach is cluster analysis, an example of which is the so-called "dendogram" used to define surface antigen CD (cluster of differentiation) groups (Spiegelhalter and Gilks, 1987). Cluster analysis often derives many clusters from a single body of data without clear biological significance and requires further interpretation and statistical validation (Hand, 1981).

In the second situation common in diagnostic pathology we are more interested in assigning cases to existing groups of known biological significance, to guide the clinicians in the appropriate patient management. The technique of discriminant function analysis (Hand, 1981) is valuable here in the assignment of cases to prognostic categories in a powerful way by deriving a set of classifiers which assign groups at a high level of probability, usually set at 95%. For each case a probability of allocation to a particular class is given and this is a valuable aid in the determination of treatment. The original data sets need to be based on a sizeable group of cases in most instances but new cases can be introduced at any time into the analysis and allocated accordingly with the additional gain of improvement of the discriminant. This approach can be used in certain diseases to define responders to therapy. The treatment can then be given only to the potential responder group, thus avoiding unnecessary toxic side effects in non-responders.

APPLICATIONS

Histopathological Diagnosis

The application of quantitative techniques to studies of pathological changes in histological sections of human tissues has been in use for over three decades. However the utilisation of such methods has been largely confined to research problems and pathophysiological situations, e.g., placental insufficiency (Aherne and Dunnill, 1966) or emphysema (Weibel, 1973). Only in the area of bone disease have the techniques been used with any regularity and achieved a place in standard diagnostic practice (Schenk, 1979). Now that relatively powerful benchtop computers are available at low cost and good software is obtainable, there is a growing interest in diagnostic applications.

It is no surprise that there has been a great concentration on the measurement of morphological features in common cancers, especially in areas where treatment regimes are improving and becoming highly selective. Studies have mainly focused on the extraction of data from routinely prepared sections, in particular nuclear size, shape and densities (Baak *et al.*, 1990; Kunze *et al.*, 1989) and in some cases chromatin texture within nuclei (Spina *et al.*, 1992). The variations in such characteristics, as expressed in the standard deviation or coefficient of variation, can also be of great value (Skinner and Whitehead, 1976; Seshadri *et al.*, 1985). The more sophisticated investigators have examined the relationships of cells and, in particular, the organisation into organoid structures (Skinner and Whitehead, 1976; Bataille *et al.*, 1986). This is a particularly difficult and time-consuming problem as fully automated methods have yet to be devised. Given the difficulty in classification it is not surprising that the non-Hodgkin's lymphomas have featured quite frequently in this literature (Link *et al.*, 1989; Ricco *et al.*, 1989; Sokol *et al.*, 1990; Tosi *et al.*, 1990). The areas of breast and ovarian cancer, in particular, have been the subject of intensive interest but there has also been a considerable attention given to bladder cancers and to epithelial tumours throughout the gastrointestinal tract (Carter *et al.*, 1989; Collins *et al.*, 1989; Lindholm *et al.*, 1989; Martin *et al.*, 1989; Schipper *et al.*, 1989a, 1989b; Umbricht *et al.*, 1989; van Diest *et al.*, 1989; Ambros *et al.*, 1990; Bibbo *et al.*,1990; Blomjous *et al.*, 1990; Miller *et al.*, 1990; Beerman *et al.*, 1991; Sowter *et al.*, 1991).

Studies on the oestrogen and progesterone receptor status within breast tumour tissue have centred on the relative number of cells positive for these receptors, and in more sophisticated hands, the amount of receptors present (Charpin *et al.*, 1989; Colley *et al.*, 1989; Horsfall *et al.*, 1989; McClelland *et al.*, 1990). Other indicators used to classify material have included the measurement of nucleolar organiser regions (Ruschoff *et al.*, 1990; Bockmuhl *et al.*, 1991), and more recently the detection and measurement and even quantitation of oncoprotein expressions with *in situ* hybridisation techniques have been shown to be of grading and prognostic value (van Diest *et al.*, 1991).

In the area of ovarian cancer the value of morphometric techniques is probably even better established than in breast tumours. The analysis of various nuclear and cytological features including the DNA content of nuclei has been widely used and shown to provide reproducible and accurate prognostic information in addition to that normally available to the pathologist (Iversen and Laerum, 1985; Baak *et al.*, 1988; Schipper *et al.*, 1989a, 1989b; Miller *et al.*, 1990).

Nuclear Studies

One of the now widely used diagnostic applications of VIA is the analysis of nuclear DNA in cytological preparations from suspected malignancies obtained by fine needle aspiration (FNA). Cell nuclei can be selectively stained by the Fuelgen method (by a stoichiometric dye for DNA) revealing discrete, magenta objects surrounded by a clear zone of unstained cytoplasm and interstitium. These discrete objects are then easily measured by VIA. The IOD of the nuclear image is directly proportional to the nuclear DNA content, thus making the measurements of DNA in the smears quantitative. This is comparable to that of flow cytometry using DNA binding fluorescent dyes, though there is less statistical precision than that available from the measurement of large numbers of cells by flow cytometry. With modern image analysis equipment it is possible to measure the DNA content of cells to a sufficiently accurate degree which usually involves measurements of several hundred cells. This level of VIA analysis usually does give reliable data and new forms of mathematical analysis are enabling more information to be extracted from VIA ploidy profiles, a histogram presentation of IOD values of DNA content. The measurement of relative DNA content within cell populations of breast tumours has been shown to be a valid and reliable prognostic index and a fairly powerful discriminator of good or bad prognostic index and a fairly powerful discriminator of good or bad prognostic categories for patients (Bocking *et al.*, 1989; Troncoso *et al.*, 1989; Wilbur *et al.*, 1990, Uyterlinde *et al.*, 1991). In flow cytometry cells of interest are diluted by large number of normal cells extracted at the same time as tumour cells and significant information is missed despite the availabilty of good gating procedures for cell size, nuclear shape and fluorescence output. VIA measurement however overcomes these problems in that cells can be studied in a particular tissue context and other nuclear or cellular features of possible diagnostic or prognostic significance measured at the same time, e.g., nuclear area and perimeter, cell shape, size and distribution, and the relative number of mitoses (Baak *et al.*, 1988; Baak and van Diest, 1989).

It is becoming clear that other cell characteristics are of diagnostic value even if they do not relate to basic biological changes within cells. These include the VIA measurement of mitotic index, proliferative assay and proliferation antigens. Mitotic index and proliferation assay using either the recognition of mitoses per "high power field" or indicators of proliferative activity such as ^{3}H-thymidine or BrdU uptake are of limited use in clinical histopathology either because of crude observation or the requirement of fresh viable tissue. For these reasons the immunohistochemical examination of proliferative antigens such as Ki-67(G2/M phase), PCNA(G1/S phase) or C5F10(M phase) are increasingly represented in the literature (Charpin *et al.*, 1989; Schwartz *et al.*, 1989; Benjamin, 1991; Caulet *et al.*, 1991; Soyer, 1991).

Immunohistochemistry

The VIA technology enables automated and objective measurements of immunohistochemically stained cells in tissue sections and this is responsible for much of the recent interest in VIA as a tool in diagnosis and research. For this application high resolution colour systems are to be preferred over the monochrome systems. For VIA measurement the immunohistochemical staining must provide: 1) sufficient contrast between the stained cells and background for the video camera to resolve, and 2) uniformity of staining across the fields.

To produce optimal contrast heavy metal enhancement variants of the immunoperoxidase methods should be used (Hsu and Soban, 1982) although standard methods with relatively faint staining can be analysed using specific waveband filters to increase the contrast. In our hands we find nickel enhancement of DAB staining in ABC immunoperoxidase with faint methyl green counterstain produces a high-contrast image. Though silver enhancement of DAB gives the highest contrast reaction the linear relationship with antigen concentration is lost. The sensitivity of the nickel-enhanced technique is 7–10 fold greater than the standard non-enhanced methods of DAB staining when using cell suspensions, comparable to high sensitivity flow cytometry (Zola *et al.*, 1990; Coventry, 1991; Coventry *et al.*, 1993). Good contrast and uniformity of reaction products in immunostaining can be reliably expected if the requirements for tissue preparation, section processing and staining conditions mentioned in the quality control section are met.

A common requirement in immunopathology is to estimate the proportions of cells positive for a particular antibody in a total population of infiltrating cells. This estimate can be made by determining the fraction of area stained by antibodies to cell subsets, e.g., an anti-CD3 monoclonal, within the area stained by antibodies to the total population, e.g., an anti-CD45 antibody (Fig.5). This estimation of relative cell number relies on the assumption that the antibodies to the subsets and total population are of comparable affinity towards their respective antigens, equal efficiency in marking the cells and covering the same areas in each cell. For most applications empirical observation has revealed this to be the case (Norazmi *et al.*, 1990).

The stained cells across the section can be assessed by taking multiple sequential field measurements, revealing the relative dispersion and the interrelations between stained cells and other cell types or structures in the section. Studies of this type emphasise the importance of the topographical relationships between cells and tissue structures. Cell distribution profile (dispersion) is a better representation of the type and degree of infiltration than grading by simple visual scoring (+ to ++++) (Coventry, 1991) since there are considerable variations in cell distribution within each visual score (Fig.6).

Moreover, VIA is useful for estimating cell numbers in a field when cells are not clearly identified as discrete entities. In situations where cells touch as an alternative to laborious on-screen editing we use the total stained area divided by the average area of a cell, as given in the following formula which offers a technique of cell quantitation comparable to manual counting:

$$\text{No. cells} = \frac{\text{Total area stained}}{\text{mean area of cell stained}}$$

This formula is based on the assumptions which hold true under most conditions: 1) reaction product is proportional to monoclonal binding and hence cell surface antigen; 2) reaction products per cell is relatively uniform and non-diffusable; 3) cell size is uniform within close limits.

Autoradiography

The measurement of *in situ* hybridisation by VIA technique gives a more precise representation than visual scoring and less labour intensive in that it is quick, automatic and semi-quantitative. This not only permits assessment of numbers and locations of

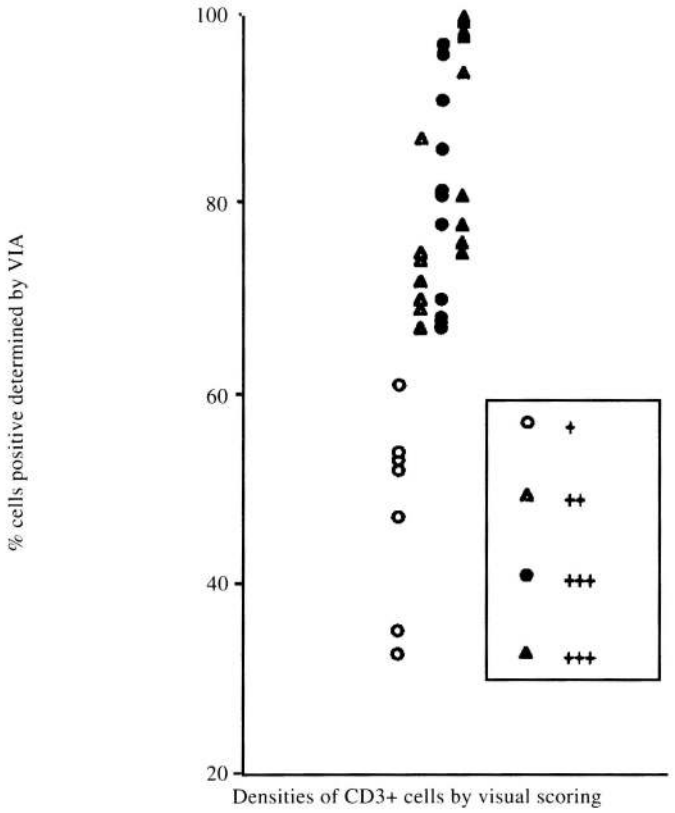

Fig. 5. Comparison of VIA and visual analysis in the estimation of immunoperoxidase stained CD3 T cells from colonic carcinoma. The results obtained with visual estimates (X axis) and VIA measurements (Y axis) show a correlation but with considerable overlap, especially at the higher levels. The visual estimates are not reliable.

positive cells but also the relative amount of nucleotides produced per cell by granule counting. When peripheral blood leucocytes were examined for IL-2 mRNA by *in situ* hybridisation using ^{35}S labelled probes, there were no differences in the percentages of positive cells between healthy young and aged, yet the IL-2 mRNA expressed per cell, as determined by the amount of deposited silver granules, was decreased in the healthy aged to only 25% of that in the young (Bradley and Xu, 1992).

CONCLUSION

The advent of computer aided video image analysis has removed to a large degree the drudgery involved in morphometric methods and the availability of relatively cheap PC platforms has brought the technology within the grasp of most diagnostic laboratories. Video cameras of high resolution and sensitivity can be found enabling quite accurate measurements to be made using colour as well as other image features as a discriminant.

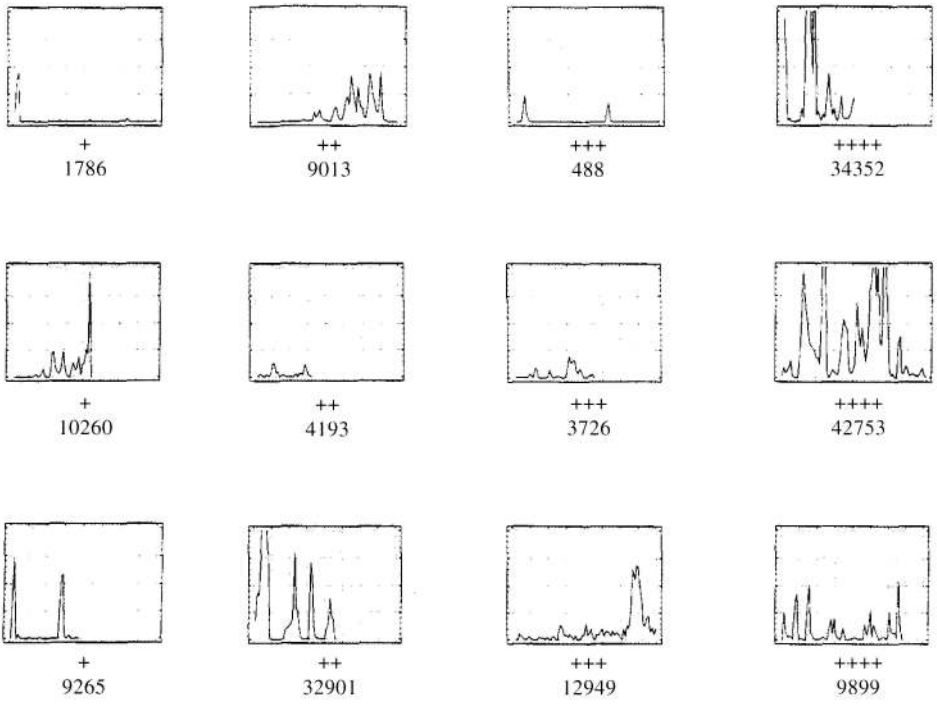

Fig. 6. VIA graphical profiles for primary breast tumour sections from 21 tumours stained with CD3 primary antibody (Leu-4). The area under the profile curve (numbers) is a more reliable estimate of cell numbers than the subjective scoring + – ++++ so often used in the literature.

This latter feature has the additional advantage of congruity with the methods of subjective analysis normally used in histopathology. The data derived from accurate measurement of tissue features are easily transferred to computer statistical packages for speedy and accurate analysis. The 'Windows'© environment now available on PCs makes this task all the easier. It is possible to make a well supported statement of probability, of a diagnosis or prognostic category for example, which can be used with other information in the construction of a report. VIA has now reached the stage that it is a sub-discipline of histopathology which can be added to and used in conjunction with, other techniques such as immunohistology to improve diagnostic practice. As with all new methods an awareness of possible pitfalls and a close attention to quality control are necessary prerequisites to success. Given the reduction in the price of hardware in the past decade we can expect that the basic platform for VIA in the medium term will move to powerful workstations such as Sun or Silicon Graphics systems, a trend already evident in the field of confocal microscopy. This will allow for the development of faster and more sophisticated software based on the Unix operating system and the introduction of useful neural networks which offer a more flexible approach than expert systems for example.

REFERENCES

Aherne, W.A.and Dunnill, M.S. (1966). Quantitative aspects of placental structure. *J. Path. Bact.*, **91**, 123–39.

Aherne, W.A.and Dunnil, M.S. (1982). *Morphometry*. London: Edward Arnold.

Ambros, R.A., Pawel, B.R., Meshcheryakov, I., Kotrotsios, J., Lambert, W.C. and Trost, R.C. (1990). Nuclear morphometry as a prognostic indicator in colorectal carcinoma resected for cure. *Anal. Quant. Cytol. Histol.*, **12**, 172–6.

Baak, J.P.A. (1990). *A Manual of Quantitative Pathology in Cancer Diagnosis and Prognosis.* Heidelberg: Springer-Verlag.

Baak, J.P.A.and Fleege, J.C. (1990). Quantitative methods in diagnostic pathology. *Verh. Dtsch. Ges. Pathol.*, **74**, 165–8.

Baak, J.P.A.and Oort, J. (1983). *A Manual of Morphometry in Diagnostic Pathology*. Heidelberg: Springer.

Baak, J.P.A., Schipper, N.W., Wisse-Brekelmans, E.C.M., Ceelen, T., Bosman, F.T., van Geuns, H. and Wils, J. (1988). The prognostic value of morphometrical features and cellular DNA content in cis-platin treated late stage ovarian cancer patients. *Br. J. Cancer*, **57**, 503–8.

Baak, J.P.A.and van Diest, P.J., (1989). Data processing and analysis in the multicentre morphometric mammary carcinoma project (MMMCP). *Path. Res. Pract.*, **185**, 657–63.

Bartels, P.H., Weber, J.E. and Duckstein, L. (1988). Machine learning in quantitative histopathology. *Anal. Quant. Cytol. Histol.*, **10**, 299–304.

Bataille, R., Chappard, D., Alexandre, C., Dessauw, P. and Sany, J. (1986). Importance of quantitative histology of bone changes in monoclonal gammapathy. *Br. J. Cancer*, **53,** 805–10.

Beerman, H., Veldhuizen, R.W., Blok, R.A., Hermans, J. and Ooms, E.C. (1991). Cytomorphometry as quality control for fine needle aspiration. A study in 321 breast lesions. *Anal. Quant. Cytol. Histol.*, **13,** 143–8.

Benjamin, D.R. (1991). Proliferating cell nuclear antigen (PCNA) and pediatric tumors: assessment of proliferative activity. *Pediatr. Pathol.*, **11**, 507–19.

Bibbo, M., Michelassi, F., Bartels, P.H., Dytch, H., Bania, C., Lerma, E. and Montag, A.G. (1990). Karyometric marker features in normal-appearing glands adjacent to human colonic adenocarcinoma. *Cancer Res.*, **50**, 147–51.

Blomjous, C.E., Vos, W., Schipper, N.W., Uyterlinde, A.M., Baak, J.P., de Voogt, H.J. and Meijer, C.J. (1990). The prognostic significance of selective nuclear morphometry in urinary bladder carcinoma. *Hum. Pathol.*, **21**, 409–13.

Bockmuhl, U., Thessig, F., Dimmer, V. and Kunze, K.D. (1991). The impact of nucleor organiser regions for the lymph node spread and prognosis of invasive ductal mammary carcinoma. P*athol. Res. Pract.*, **187**, 437–43.

Bocking, A., Chatelain, R., Biesterfeld, S., Noll, E., Biesterfeld, D. and Wohltmann, D. (1989). DNA grading of malignancy in breast cancer. Prognostic validity, reproducibility and comparison with other classifications. *Anal. Quant. Cytol. Histol.*, **11**, 73–80.

Bradley, J. and Xu, X. (1992). Monoclonal Antibodies – Fifteen Years Use in Basic and Clinical Research in Medicine. *Proceedings of the South East Asia Seminar on Immunology and Workshop on Biotechnology, Kelantan Malaysia.* 83–100.

Carter, C.D., Weeks, S.C., Jarvis, L.R. and Whitehead, R (1989). Morphometric analysis of nuclear features, ploidy status, and staging in rectal carcinoma. *J. Pathol.*, **159**, 113–20.

Caulet, S., Brousset, P., Schoevaert, D., Chittal, S.M., Delsol, G., Audouin, J. and Diebold, J. (1991). Quantitative study of KI-67 antibody staining in 46 T-cell malignant lymphomas using image analysis. *Hematol. Oncol.*, **9**, 323–35.

Charpin, C., Andrac, L., Habib, M.C., Vacheret, H., Xerri, L., Devictor, B., Lavaut, M.N. and Toga, M. (1989). Immunodetection in fine-needle aspirates and multiparametric (SAMBA) image analysis. Receptors (monoclonal antiestrogen and antiprogresterone) and growth fraction (monoclonal Ki67) evaluation in breast carcinomas. *Cancer*, **63**, 863–72.

Colley, M., Kommoss, F., Bibbo, M., Dytch, H.E., Franklin, W.A., Holt, J.A., Wied, G.L. (1989). Assessment of hormone receptors in breast carcinoma by immunocytochemistry and image analysis. II. Estrogen receptors. *Anal. Quant. Cytol. Histol.*, **11**, 307–14.

Collins, J.S., Watt, P.C., Hamilton, P.W., Collins,B.J., Sloan, J.M., Elliot, H. and Love, A.H. (1989). Assessment of oesophagitis by histology and morphometry. *Histopathology*, **14**, 381–9.

Coventry, B. (1991). Characterisation of tumour infiltrating lymphocytes in human solid tumours – using standard and video image analytic methods for cellular quantitation of immunostained cells. PhD thesis, Flinders University, Adelaide, South Australia, Australia.

Coventry, B.J., Bradley, J., Skinner, J.M. (1993). Differences between standard and high-sensitvity immunoperoxidase staining methods in tissue sections. Comparison of immunoperoxidase staining methods using computerised video image analysis techniques. *J. Histochem. Cytochem.*, submitted.

Dunnill, M.S. (1968). Quantitative methods in histology. In: Dyke, S.C. (Ed). *Recent Advances in Clinical Pathology*. London: Churchill Livingstone, 401–16.

Hand, D.J. (1981). Miscellaneous topics. Analysis of a classifier. In: Hand, D.J. (Ed). *Discrimination and Classification.* Chichester, New York: Wiley.

Horsfall, D.J., Jarvis, L.R., Grimbaldeston, M.A., Tilley, W.D., Orell, S.R. (1989). Immunocytochemical assay for oestrogen receptor in fine needle aspirates of breast cancer by video image analysis. *Br. J. Cancer*, **59**, 129–34.

Hsu, S-M.and Soban, E. (1982). Colour modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunochemistry. *J. Histochem. Cytochem.*, **30**, 1079–82.

Itoh, J., Osamura, R.Y. and Watanabe, K. (1992). Subcellular visualisation of light microscopic specimens by laser scanning microscopy and computer analysis: a new application of image analysis. *J. Histochem. Cytochem.*, **40**, 955–67.

Iversen, O.E.and Laerum, O.D. (1985). Ploidy disturbances in endometrial and ovarian carcinomas. A review. *Anal. Quant. Cytol. Histol.*, **7**, 327–36.

Jarvis, L.R. (1992). The microcomputer and image analysis in diagnostic pathology. *Microsc. Res. Tech.*, **21**, 292–9.

Kunze, K.D., Haroske, G., Dimmer, V., Meyer, W. and Thessig, F. (1989). Quantitative morphology of primary and metastatic tumors: comparative studies on tissue sections. *Anal. Cell. Pathol.*, **1**, 143–51.

Lindholm, J., Rubio, C., A., Kato, Y. and Hata, J. (1989). A morphometric method to discriminate normal from dysplastic/carcinoma *in situ* squamous epithelium in the human esophagus. *Pathol. Res. Pract.*, **184**, 297–305.

Link, N.G., Nathwani, B.N. and Preston, K. Jr. (1989). Subclassification of follicular lymphomas by computerized image processing. *Anal. Quant. Cytol. Histol.*, **11**, 119–30.

McClelland, R.A., Finlay, P., Walker, K.J., Nicholson, D., Robertson, J.F., Blamey, R.W., and Nicholson, R.I. (1990). Automated quantitation of immunocytochemically localized estrogen receptors in human breast cancer. *Cancer Res.*, **50**, 3545–50.

Martin, H., Beil, M., Hufnagl, P., Wolf, G. and Korek, G. (1989). Comparison of light microscopic grading and morphometric features in cytological breast cancer specimens. *Pathol. Res. Pract.*, **185**, 612–6.

Miller, B.E., Lavia, L.A. and Horbelt, D.V. (1990). The prognostic value of image analysis in ovarian cancer. *Cancer*, **67**, 1318–21.

Norazmi, M.N., Hohmann, A.W., Jarvis, L.R., Skinner, J.M., Stoll, P. and Bradley, J. (1990). The use of the computer-assisted video image analysis in the enumeration of immuno-stained cells in tissue sections. *J. Immunol. Methods,* **131**, 223–7.

Ooms, E.C., Anderson, W.A., Alons, C.L., Boon, M.E. and Veldhuizen, R.W. (1983). Analysis of the performance of pathologists in the grading of bladder tumours. *Hum. Pathol.*, **14,** 140–3.

Ricco, R., Lison, V., Specchia, G., Pavone, V., Marzano, N., Lettini, T., Pesce-Delfino, V. and Pennella, A. (1989). Hand-mirror (HM) cells in acute lymphoblastic leukaemia (A.L.L.). An attempt at cellular classification by morphometric analytical parameters using the S.A.M. (Shape Analytical Morphometry) system. *Pathol. Res. Pract.*, **185**, 715–8.

Ruschoff, J., Neumann, K., Contractor, H., Plate, K., Thomas, C. (1990). Assessment of nucleolar organiser regions by automatic image analysis in breast cancer: correlation with DNA content, proliferation rate, receptor status and histopathological grading. *J. Cancer Res. Clin. Oncol.*, **116**, 480–5.

Schenk, R.K. (1979). Model cases from biological morphometry. Bone tissue. In: Weibel, E.R. (Ed). *Stereological Methods, Vol I,*. 301–11.

Schipper, N.W., Smeulders, A.W. and Baak, J.P. (1989a). Evaluation of automated estimation of epithelial volume and its prognostic value in ovarian tumors. *Lab. Invest.*, **61**, 228–34.

Schipper, N.W., Smeulders, A.W., de Lange, J.H. and Baak, J.P. (1989b). Quantification of epithelial area by image processing applied to endometrial carcinomas: a comparison with ovarian tumors. *Hum. Pathol,* **20**, 1125–32.

Schwartz, B.R., Pinkus, G., Bacus, S., Toder, M. and Weinberg, D.S. (1989). Cell proliferation in non-Hodgkin's lymphomas. Digital image analysis of Ki-67 antibody staining. *Am. J. Pathol.*, **134,** 327–36.

Seshadri, R., Jarvis, L.R., Jamal, O. and Skinner, J.M. (1985). A morphometric classification of acute lymphoblastic leukemia in children. *Med. Pediatr. Oncol.*, **13**, 214–20.

Skinner, J.M.and Whitehead, R. (1976). Morphological methods in the study of the gut immune system in man. *J. Clin. Path.*, **29**, 564–7.

Sokol, R.J., Hudson, G., James, N.T. and Wales, J. (1990). Morphometry of macrophage development in malignant lymphoma. Predictive value of discriminant analysis. *Vircows Arch. B.*, **58,** 185–8.

Sowter, C., Slavin, G., Sowter, G., Rosen, D. and Hendry, W. (1991). Morphometry of bladder carcinoma: morphometry and grading complement each other. *Anal. Cell. Pathol.*, **3,** 1–9.

Soyer, H.P. (1991). Ki 67 immunostaining in melanocytic skin tumors. Correlation with histologic parameters. *J. Cutan. Pathol.*, **18**, 264–72.

Spiegelhalter, D.J.and Gilks, W.R. (1987). Statistical Analysis. In: McMichael, A.J. (Ed). *Leucocyte Typing III.* Oxford, 14–24.

Spina, D., Disanto, A., Luzi, P., Tosi, P., Gallorini, M., Mouthon, A.M., Kraft, R. and Cottier, H. (1992). Novel, contrast gradient-oriented, automatic chormatin texture analysis. I.. Feasibility study on nuclei from benign and malignant breast epithelial cell lines in fine needle aspirates. *Virchows Arch. B. Cell. Pathol.*, **62**, 119–24.

Tosi, P., Leoncini, L., Del-Vecchio, M.T., Cevenini, G., Stramignoni, A., Valente, G., Palestro, G., Novero, D. and Cottier, H. (1990). Heterogeneous subgroups among malignant diffuse small B cell lymphomas. A combined nucleometric and immunocytologic study. *Lab. Invest.*, **62**, 202–12.

Troncoso, P., Dytch, H.E., Bibbo, M., Wied, G.L. and Dawson, P.J. (1989). The significance of DNA measurements in a histologically defined subset of infiltrating ductal carcinomas of the breast with long-term follow-up. *Anal. Quant. Cytol. Histol.*, **11**, 166–72.

Umbricht, C., Oberholzer, M., Gschwind, R., Christen, H. and Torhorst, J. (1989). Prognostic significance (relapse, non-relapse) of nuclear shape parameters in lymph node negative breast cancer. *Anal. Cell. Pathol.*, **1**, 11–23.

Uyterlinde, A.M., Baak, J.P., Schipper, N.W., Peterse, H.J., Meijer, J.W., Vooys, P.G. and Matze, E. (1991). Prognostic value of morphology and DNA flow-cytometry features of invasive breast cancers detected by population screening: comparison with control group of hospital patients. *Int. J. Cancer.,* **48**, 173–81.

van Diest, P.J., Baak, J.P., Chin, D., Theeuwes, J.W. and Bacus, S.S. (1989). Potential of morphometry in grading transitional cell carcinoma of the urinary bladder. *Pathol. Res. Pract.*, **185,** 617–20.

van Diest, P.J., Baak, J.P., Chin, D., Theeuwes, J.W. and Bacus, S.S. (1991). Quantitation of HER-2/neu oncoprotein overexpression in invasive breast cancer by image analysis: a study comparing fresh and paraffin-embedded material. *Anal. Cell. Pathol.*, **3**, 195–202.

Weibel, E.R. (1973). A simplified morphometric method for estimating diffusing capacity in normal and emphysematous human lungs. *Am. Rev. Resp. Dis.*, **107**, 579–88.

Weibel, E.R. (1980). Fundamental operations in stereology. In: Weibel, E.R. (Ed). *Stereological Methods, Vol. 2*. London: Academic Press, 19–54.

Weid, G.L., Bartel, P.H., Bibbo, M. and Dytch, H.E. (1989). Image analysis in quantitative cytopathology and histopathology. *Hum. Pathol.*, **20**, 549–71.

Wilbur, D.C., Zakowski, M.F., Kosciol, C.M., Sojda, D.F. and Pastuszak, W.T. (1990). DNA ploidy in breast lesions. A comparative study using two commercial image analysis systems and flow cytometry. *Anal. Quant. Cytol. Histol.*, **12**, 28–34.

Zola, H., Neoh, S.H., Mantzioris, B.X., Webster, J., Loughnan, M.S. (1990). Detection by immunofluorescence of surface molecules present in low copy numbers. High sensitivity staining and calibration of flow cytometer. *J. Immunol. Methods*, **135**, 247–55.