Principles of ploidy analysis by static cytometry

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Abstract

Aims—To examine the basic assumptions made during DNA ploidy analysis of histological sections with an aim to eliminate methodological errors that have lead to conflicting results with this technique.

Methods—A rat liver imprint and histological sections together with sections and whole nuclei cytopspins of human breast tumour biopsy specimens were stained with azure A Schiff's reagent and used to investigate the effects of computer imaging, histological section thickness, nuclear volume, and shape corrections.

Results—The rat liver imprint demonstrated a linear relation between mean nuclear transmittance and nuclear area for each of the three (2C, 4C, 8C) hepatocyte clusters. This finding was used to produce similar, proportional integrated optical density (IOD) measurements from rat liver sections by selecting only nuclei that were sectioned through their centres, as assumed by the mathematics for volume correction. The limitations of computer imaging necessitated an edge (glare) correction for each nucleus so that nuclei of different sizes could be analysed. Shape correction was required to analyse nuclei of different morphologies. Normal human lymphocytes, squamous epithelium and fibroblasts were all measured with similar IODs. DNA ploidy values obtained from 7 µm human breast tumour sections (using lymphocytes as controls) correlated well with those obtained from the whole nuclei cytopspins from the same tissue blocks.

Conclusions—With an improved understanding of the theoretical and technical aspects of ploidy analysis of tissue sections, reproducible and consistent results are possible. These results can be integrated into routine histopathology investigations alongside immunohistochemistry and molecular diagnostic techniques.

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Quantitative DNA (ploidy) analysis is a measure of the amount of DNA in cell nuclei. During the transformation from normality to malignancy a number of alterations to the DNA take place.^{1,2} Analysis of the overall amount of DNA that is present in a population of cells can indicate the degree of genetic damage that the cells have sustained.³

Ploidy analysis can be used as a prognostic indicator. The degree of genetic damage has been correlated with tumour outcome and many studies have concluded that, in general

terms, aneuploidy (an abnormal amount of DNA) is associated with a worse prognosis than diploidy.⁴⁻⁶ However, it has been suggested that the more appropriate use of ploidy analysis lies not with poorly differentiated, aggressive (aneuploid) tumours, but with well differentiated, low grade tumours or dysplastic conditions, or both, where outcomes are less certain.^{7,8}

There are two techniques which are used to perform DNA quantitation: flow cytometry and static cytometry. The former analyses a large number of nuclei from whole cell preparations using a flow cytometer. This technique has the advantage of being relatively quick, but "rare events" (that is, specific cell clones) can be hidden amongst the data.² Static cytometry analyses small numbers of nuclei in tissue sections or cytological smears using a light microscope and image analyser. Data collection times have been greatly reduced with faster computers and the method has the advantage that it is combined with morphology assessments, permitting visualisation (and therefore selection) of specific nuclei for analysis. A dye is bound stoichiometrically to the DNA and the amount of dye is then analysed to estimate the ploidy status of the cell population. While a fluorescent dye is used for flow cytometry, two separate families of dyes have been used for most static cytometric investigations. Basic fuchsin and azure A/thionin have been made into a Schiff's reagent and coupled to the deoxyribose ring of the DNA after hydrolysis with HCl.^{7,9-11} Densitometric analysis is then carried out and the optical density of each nucleus is used to indicate the amount of DNA present.

However, in recent years, conflicting findings have resulted in controversy and a decline in the use of ploidy analysis. A number of reports have presented opposing views on the correlation between ploidy and tumour outcome.^{2,6} Some of this conflict may have resulted from attempts to correlate only the ploidy findings with tumour outcome, and it is now increasingly recognised that other biological variables such as the proliferation index, oncogene and suppressor gene expression, hormone receptor status, and other immunohistochemical indexes should also be considered in this assessment.¹²⁻¹⁴ Perhaps the main reason for this controversy is a failure to understand the basic principles of ploidy analysis.

Many of the sources of errors which have lead to conflicting results are based on methodology.^{15,16} Ploidy analysis is a mixture of a number of disciplines including histology, computer imaging, stereology, and densitometry. The effects of variables within each discipline such as different staining dyes, the

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choice of control cell nuclei and the sizes and shapes of nuclei have not been investigated fully. Many basic assumptions are made during the analysis procedure and it is essential that these are valid if reliable results are to be obtained.

A complete understanding of the principles of ploidy analysis has not been readily available. Whilst the concept of binding a dye stoichiometrically to the DNA and performing densitometric analysis seems to be straightforward, the details of this methodology are either ignored or not disclosed by most authors. The larger commercial systems seem to produce dependable results, but the way in which the nuclei are selected for analysis can be complex. For example, some systems use elaborate matrix transformations for each pixel which are not easily transferred to smaller, "stand-alone" analysers.¹⁰ There have been many excellent papers which have used rat liver to demonstrate the accuracy of various analytical procedures,¹⁷⁻¹⁹ but rat hepatocytes are very different from human tumour specimens. A large number of additional technical challenges await the investigator when moving from rat liver to human tumour analysis.

Procedures within the various methodologies need to be standardised.^{6,15,20} The staining method, section thickness, computer imaging, analysis, data handling, and interpretation all need to be consistent and reproducible so that results from different laboratories can be compared effectively.

The aim of this paper is to examine and analyse the many basic assumptions that are made when performing ploidy analysis on tissue sections. In so doing it is hoped that a more complete understanding of the principles of ploidy analysis will allow investigators to obtain more comparable results.

Methods

RAT LIVER IMPRINT

An imprint of a rat liver was made on a glass slide. This was air dried briefly, fixed in neutral buffered formalin for 30 minutes, and the nuclei stained by the azure A Feulgen method. Hydrolysis was performed for one hour at room temperature using 5 N HCl, followed by one hour in the Feulgen stain. The slide was then washed three times over 10 minutes in the rinse solution, placed in running tapwater for a further 10 minutes and finally immersed in acid/alcohol for five minutes before dehydration, clearing and mounting.

The azure A staining solution was prepared by mixing 50 ml 1% concentrated HCl, 1 g potassium metabisulphite ($K_2S_2O_5$) and 0.25 g azure A in a sealed container for one hour at room temperature. This solution was filtered into a Coplin jar immediately before use. The rinse solution was made by mixing 200 ml 0.05% concentrated HCl and 2 g $K_2S_2O_5$.

The stained nuclei were analysed using a Tracor Northern TN8500 image analyser which was connected to a Mintron CCD black and white camera mounted on an Olympus BH-2 light microscope. A narrow band in-

terference filter was used to produce red light with a wavelength of 630 nm and the analysis was carried out using a 40× SPlan apochromatic objective lens with the condenser set at 0.9, which is the numerical aperture of this lens.

The dynamic range (that is, the range of grey levels) of the image was maximised to ensure the greatest sensitivity and the camera was confirmed as having a linear response using Kodak Wratten gelatin filters. A correction table was set up using the black (0% light) and white (100% light) levels and this was applied to each image.

The threshold was set so that the nuclei were completely covered and the integrated optical density (IOD) was calculated for each nucleus by multiplying the number of pixels by the $-\log$ of its mean transmittance. The latter was calculated mathematically by dividing the pixel values from a blank, background image into each image containing nuclei. This procedure was also an effective shade correction. However, because of the inherent problems of CCD camera imaging (glare), an edge correction was applied during the IOD calculation. This correction involved the erosion of two layers of pixels from the binary image of each nucleus before the mean transmittance and the area were measured. However, the original area (in pixels) was needed to calculate the IOD and this was derived mathematically by determining the radius of the eroded area, adding two pixels and recalculating the original area of each nucleus. This calculation assumed the nuclei were circular in profile (spherical in shape).

The raw data were collected using the TN 8500 image analyser and this was transferred to a PC for subsequent data handling using an Excel spreadsheet (Microsoft Corporation). Two hundred nuclei were analysed and the result was visualised as an IOD/nuclear area scatter plot. The mean and standard deviation of the IOD values were calculated for each of the 2C, 4C and 8C clusters. A mean nuclear transmittance/area scatter plot was also obtained.

RAT LIVER SECTIONS

A portion of the same rat liver was processed for routine histology, sections were cut at thicknesses of 3, 5 and 7 μ m, and stained as outlined earlier. The threshold was set so that most of the nuclei were covered, but because of partial sectioning it was not usually possible to include the whole of every nucleus without incorporating part of the surrounding cytoplasm. A simple holefill, erode and dilate routine was used to fill "holes" in nuclei, remove small, unwanted particles and to separate touching nuclei automatically. A shape filter for circularity ($(\text{perimeter})^2 / (4\pi \times \text{area})$) was applied during data collection to reject irregular or groups of nuclei. Nuclei with circularities of less than 1.5 were accepted for analysis; the circularity of a circle is 1.

Because of the variation in nuclear sizes, some nuclei were included completely within the section whilst others were partially cut.

To allow for this inequity the IOD value was corrected depending on the nuclear size. If the diameter of a nucleus was less than the section thickness, the IOD value was not modified. However, if the nuclear diameter was greater than the section thickness, the modified equation of McCready and Papadimitriou²¹ was used to calculate the fraction of nucleus within the section and the corrected IOD calculated by dividing the observed IOD by the nuclear fraction shown in box 1.

Box 1

Spherical nuclear fraction = $\frac{3T}{4R} - \frac{T^3}{16R^3}$

where T = section thickness and R = nuclear radius.

Nuclei that were sectioned through their centres were selected for final analysis. The above equation for the fraction of nucleus in the section assumes that the plane of section passes through the centre of the nucleus. The information obtained from the transmittance/area scatter from the imprint was used to formulate a procedure to exclude those nuclei that were not sectioned through their centres. This exclusion process was performed by an Excel macro routine. The coordinates of two points were entered which defined a line through the top of each transmittance/area cluster. Using the simple equation for a straight line ($y = ax + b$), the macro routine calculated the y (area) intercept and the slope of the line, a. All points in the transmittance/area cluster lying between the x (transmittance) values of the two points and lying along a line with a slope of a $\pm 15\%$ were selected for analysis. Nuclei outside this range were considered not to be sectioned through their centres and were excluded.

A minimum of 300 nuclei was analysed from each section to ensure that at least 200 were considered in the final analysis. The transmittance/area and IOD/area scatter plots were obtained, together with the mean, standard deviation and coefficient of variance for each of the 2C, 4C and 8C clusters. These data were tabulated together with the results from the imprint.

HUMAN BREAST SECTIONS

The effect of nuclear shape was addressed by measuring the aspect ratio of each nucleus. If this was less than 1.5 then the nucleus was considered to be spherical and the edge and IOD corrections were carried out as before. However, if the aspect ratio was greater than 1.5, the nucleus was assumed to be a sausage shape. In this case, the edge correction was performed with the same two-pixel erosion, but the mathematical calculation of the original nuclear area was carried out by multiplying the external perimeter (in pixels) by two and adding this to the eroded nuclear area. The calculation of the fraction of nucleus in the section was

also modified for sausage shaped nuclei. These nuclei were assumed to be two half spheres connected by a short central cylinder and oriented so that the major axis of the nucleus is parallel to the plane of section. The width (minor axis) of the nucleus is therefore the critical dimension in determining the fraction of nucleus in the section. This was calculated by adding the fraction of the central cylinder present to an expanded form of the above equation for the fraction of a sphere (box 2).

Box 2

Sausage nuclear fraction = $\frac{[\text{volume of sphere in section}] + [\text{volume of cylinder in section}]}{[\text{total volume of sphere}] + [\text{total volume of cylinder}]}$

Sausage nuclear fraction =
$$\frac{\left[\pi r^2 T - \left(\frac{\pi}{3} \times \frac{T^3}{4}\right)\right] + \left[(R-r) \times \left(2r^2 \arcsin \frac{T}{2r}\right) + \left(T \times \sqrt{r^2 - \left(\frac{T}{2}\right)^2}\right)\right]}{\left[\frac{3\pi r^3}{4}\right] + [(R-r) \times \pi r^2]}$$

where r = nuclear width/2, R = nuclear length/2, and T = section thickness.

Sections, 7 μm thick, were cut from a block of routinely processed breast tissue which contained normal squamous epithelium, clusters of lymphocytes and fibroblasts. A number of nuclei from each of these cell types was analysed and the effect of each type of correction (spherical or sausage) was assessed.

Lymphocytes were used as diploid control cells. We decided to work in DNA ploidy units where a diploid nucleus had a value of 2, rather than a DNA index of 1. The average IOD value for the lymphocyte nuclei was divided into the IOD values of all other nuclei and multiplied by two to give the ploidy value of each nucleus. These were plotted as ploidy/area scatter graphs.

Four breast lesions were analysed. One case of epithelial hyperplasia and three cases of infiltrating duct carcinoma graded I, II and III (Bloom and Richardson) were selected from our archives. Sections, 7 μm thick, were cut from routinely processed paraffin wax blocks and stained as outlined earlier. The area, external perimeter, maximum length, width, and aspect ratio were measured for each nucleus. An Excel macro then carried out the edge correction and calculated the IODs based on the nuclear size and shape. Transmittance/area scatter plots were produced, coordinates entered into the macro and the inappropriately sectioned nuclei were excluded from the data set. The remaining IOD values were then converted to ploidy values using lymphocyte control data.

Cytospins were prepared from the same paraffin wax blocks, stained and analysed in the same way as the rat imprints. These were obtained in the following manner. Sections, 50 μm thick, were cut from each block, dewaxed, hydrated, digested with pepsin at a

Figure 1 Rat liver imprint showing (A) IOD/area scatter plot with central line and $\pm 15\%$ limits overlaying the 4C cluster, and (B) transmittance/area scatter plot.

pH of 1.5,²² cytopun, and stained. Data were tabulated with the results from the histological sections and linear regression analysis performed to determine whether there were differences in the DNA ploidy values from these two analytical methods.

Results

RAT LIVER IMPRINT

Figure 1 shows the IOD/area and transmittance/area scatter plots for the rat liver imprint. There were three distinct clusters in each graph corresponding to the 2C, 4C and 8C hepatocytes. The IOD graph (fig 1A) showed the clusters to be in proportion with each other. The transmittance graph (fig 1B) demonstrated that there was a linear relation between trans-

mittance and area for each of the hepatocyte groups. The line through the centre of the 4C transmittance/area cluster shows that the $\pm 15\%$ slope limit was sufficient to include all the nuclei in this group.

RAT LIVER SECTIONS

Figure 2A illustrates the transmittance/area plot for rat liver sectioned at $7\ \mu\text{m}$. Three separate clusters were visible, the top edges of which were straight lines similar to that seen with the imprint. These data points represented nuclei, irrespective of size, which had been sectioned through their centres. However, there was a scatter of points to the right and down from the top edge of each cluster. These scattered points represented nuclei that were not sectioned through their centres. The scatter to the right resulted from the inclusion of a lesser amount of DNA (that is, nuclear volume), which allowed the transmittance to increase. The scatter downwards resulted from a decrease in nuclear area as the plane of section moved away from the centre of the nucleus.

Selection of the top of each cluster eliminated inappropriately sectioned nuclei. A line and the $\pm 15\%$ selection limits can be seen overlaying the 4C cluster (fig 2A). Figures 2B–D show the effect of this selection procedure on all three clusters in both the transmittance/area and IOD/area plots for the $7\ \mu\text{m}$ section. The transmittance/area scatter of the selected nuclei looks similar to that seen with the imprint (fig 1B). Figure 2C shows the IOD/area plot of all the nuclei without applying any corrections. There is scatter to the left of the main clusters which are nuclei not sectioned through their centres. In addition, the IOD values of the main clusters are not in proportion as there has been no volume correction for nuclear size. Figure 2D shows the same data after selection for appropriately sectioned nuclei and applying

Figure 2 Rat liver section, $7\ \mu\text{m}$ thick, showing (A) transmittance/area plot of all nuclei with selection limits overlaying the 4C cluster, (B) transmittance plot with inappropriately sectioned nuclei removed, (C) and (D) IOD/area plots before and after selection and volume correction of nuclei.

Table 1 IOD values for rat liver imprint and sections. Mean $\pm$ SD (CV%)

	2C	4C	8C
Imprint	427 $\pm$ 34 (8.1)	850 $\pm$ 55 (6.6)	1609 $\pm$ 148 (9.2)
Section			
3 μ m	315 $\pm$ 55 (15.2)	686 $\pm$ 97 (14.2)	1588 $\pm$ 244 (15.4)
5 μ m	336 $\pm$ 31 (9.3)	715 $\pm$ 77 (10.8)	1554 $\pm$ 158 (10.2)
7 μ m	336 $\pm$ 21 (6.4)	700 $\pm$ 32 (4.7)	1412 $\pm$ 129 (9.2)

Table 2 The effect of full shape correction *v* simple spherical correction on the IOD values for nuclei of differing size and shape. Mean $\pm$ SD (CV%)

	Lymphocytes	Epithelial	Fibroblasts
Full corrections	338 $\pm$ 25 (7.3)	354 $\pm$ 40 (11.3)	361 $\pm$ 39 (10.8)
Spherical correction	343 $\pm$ 17 (5.0)	344 $\pm$ 48 (14.1)	306 $\pm$ 30 (9.8)

volume corrections, showing three discrete clusters which are in proportion.

All three sections produced the same result. The mean, standard deviation and coefficient of variance (CV) of the IOD values for each cluster in each section and imprint are summarised in table 1. The IOD values for each section were the same and the proportionality of the clusters was maintained as for the imprint. It is also interesting to note that as the section thickness was reduced, the CVs of each cluster became larger.

HUMAN BREAST TISSUE

The effect of nuclear shape correction is shown in table 2. The use of a simple spherical correction did not apply well to more elongated cells such as fibroblasts. This resulted in underestimation of the nuclear area and therefore the IOD of fibroblast nuclei. When the full sausage correction was applied to the same

data, a corresponding increase in the IOD values for the fibroblast nuclei was seen, aligning the IOD of these cell types ($\pm 10\%$) with the other normal, diploid lymphocytes and epithelial cells.

Figure 3 shows the transmittance/area and DNA ploidy/area scatter plots for the grade III (aneuploid) tumour. The main cluster of tumour nuclei can be seen with a line through its centre and the $\pm 15\%$ selection limits (fig 3A). The result of this selection is seen in fig 3B, which shows the diploid lymphocyte and the aneuploid tumour clusters.

A summary of the data from the 7 μ m sections and cytopspins of the four breast lesions is shown in table 3. The average IOD value for the control lymphocytes was used to convert IOD values to DNA ploidy values in each instance. There was a good statistical correlation between the results from the sections and cytopspins from each lesion with a correlation coefficient of $r=0.991$ and $p<0.001$.

Discussion

We have shown that by understanding the basic assumptions of this methodology, both accurate and reproducible ploidy analysis can be obtained from tissue sections. Using the correct dye to stain the sections, and by addressing the problems associated with CCD camera imaging and the stereological challenges of volume and shape corrections, correct results are achievable with a basic image analyser coupled to a spreadsheet.

DNA ploidy analysis has been used to detect changes in nuclear DNA for many years.⁵ Researchers have used this technique to analyse both cultured cells and tumour biopsy specimens. Ploidy analysis of tumours has been used as a prognostic marker and, in general, has indicated that aneuploidy is associated with a worse prognosis than diploidy, but the relation is not simple.^{4,6} Many other biological factors such as proliferation index, oncogene and suppressor gene expression, and immunohistochemical markers can also be used predict outcomes, so quantitative DNA analysis should be seen as one component of a set of prognostic indicators.^{13,14}

Static cytometry has been used increasingly in recent years and has the advantage of analysing "rare events". Most DNA ploidy analysis has been, and still is, carried out using flow cytometry, which has the advantage of being relatively quick and can be used to analyse a large number of cells. However, it is not possible to visualise what is being analysed and so the results from "rare events" (for example, specific clones) can be hidden amongst the main data. Static cytometry combines morphology, permitting the careful selection and analysis of specific cells. This is important as numerous genetic insults are required for the establishment of many malignancies¹² and being able to analyse subgroups of tumour cells (for example, cells which have become invasive rather than carcinoma in situ) may hold the key to prognosis and understanding of tumorigenesis.

Figure 3 Grade III aneuploid tumour showing (A) transmittance/area plot with lymphocytes (*) and the main tumour cluster with a central line and $\pm 15\%$ selection limits, and (B) DNA ploidy/area scatter of the selected nuclei.

Table 3 Comparison of results from cytopins and sections of breast lesions

	Cytospin		7 µm section	
	n	DNA ploidy (CV%)	n	DNA ploidy (CV%)
Epithelial hyperplasia	c 173	2.0 ± 0.15 (7.4)	51	2.0 ± 0.1 (4.8)
	t 175	2.01 ± 0.21 (10.4)	152	2.0 ± 0.17 (8.6)
Grade I	c 101	2.0 ± 0.13 (6.6)	28	2.0 ± 0.13 (4.8)
	t ₁ 62	2.18 ± 0.18 (8.1)	71	1.87 ± 0.24 (13.0)
	t ₂ 101	3.95 ± 0.33 (8.3)	100	3.99 ± 0.57 (14.0)
Grade II	c 85	2.0 ± 0.13 (6.5)	41	2.0 ± 0.11 (5.7)
	t 113	3.59 ± 0.28 (7.8)	143	3.92 ± 0.47 (11.9)
Grade III	c 45	2.0 ± 0.17 (8.7)	42	2.0 ± 0.13 (6.3)
	t 192	5.46 ± 0.4 (7.3)	114	5.52 ± 0.69 (12.6)

n = number of nuclei; c = control lymphocytes; t = tumour.

More recently, conflicting results have led to a decline in the use of ploidy analysis. A large number of both flow and static cytometric studies have been reported, but unfortunately, the conclusions from many of these have been conflicting.^{2,6} This may, in part, be because of the limitations of correlating only ploidy with tumour outcome. However, a large part of this controversy may be the result of not fully understanding the technical difficulties associated with ploidy analysis.

Many of the problems causing these conflicts can be traced to methodological errors.^{15,16} DNA ploidy analysis of tissue sections involves a combination of histology, stereology and densitometry. Each of these disciplines is fraught with technical challenges and when combined into one procedure, the inadequacies of one area are compounded by short-falls in another, so that the end result can be erroneous.

Azure A dye was used to stain the nuclear DNA in this study. Both red Schiff's (basic fuchsin) and blue Schiff's (azure A) have been used extensively for ploidy analysis. Both dyes are assumed to bind to the deoxyribose ring of the DNA and therefore indicate the amount of DNA present. However, the use of basic fuchsin Schiff's reagent produced a skew in the IOD/area scatter plots.^{7,9} This skew in the data resulted from residual (non-DNA bound) dye in the nuclei (unpublished data) and produced scatter plots in which the IOD is related more to the size of the nucleus than the amount of DNA present. The use of azure A in this study produced reliable and reproducible results. The important difference in these staining methods is the acid/alcohol differentiation step at the end of the staining procedure. In an acid/alcohol environment all non-DNA bound dye is removed from the section as only the azure A-DNA bond is stable.²³ It is not possible to differentiate effectively the basic fuchsin Schiff's dye.

Edge (glare) correction was essential for analysis of nuclei of different sizes. Advances in computer based image analysers have been considerable over the past 10 years. However, there are still some limitations to the imaging of dark objects. Between the dark centre and the lighter background at the edge of each imaged object are pixels with intermediate grey levels. Inclusion of these pixels in IOD calculations can cause underestimation of nuclear density. This "edge effect" is most important when considering nuclei of different sizes, as the two-pixel layer used in the edge correction

can represent up to 50% of the area of small nuclei whereas this "edge" may be only 10% of the area of larger nuclei. It is therefore essential that the edge correction be carried out so that the IOD of smaller nuclei is not underestimated. From the "backward lean" of the clusters within the IOD/area scatters produced by the CAS 200, it would seem that during the pixel matrix transformation, this instrument also considers the edge pixels.¹⁸

Both azure A and edge correction are necessary if lymphocytes are to be used as control cells. Inflammatory cells are the only cell types found in all tumour biopsy specimens. For this reason they constitute an ideal control. However, their use as control, diploid cells is controversial. Much of this dispute has arisen from differences in staining and imaging techniques. In general, authors who concluded that lymphocytes were unsuitable as controls used basic fuchsin Schiff's to stain their sections, giving large skews in their data.^{7,9} One solution to this problem is to use control nuclei of the same size as those of the tumour, but this can be difficult with larger tumour nuclei.²⁴ In addition, this procedure introduces the problem of differing fixation times between sample and control cells. Those who have used lymphocytes successfully as diploid controls have stained their sections with azure A and seem to have used an edge correction (or matrix transformations), as reported here.²⁵

Volume correction assumes that the nuclei are sectioned through their centres or "equators". There are a number of methods for calculating nuclear volume (and therefore nuclear fraction) in tissue sections.^{26,27} Some of these methods are complex and so the most popular method seems to use the equation of McCready and Papadimitriou.¹⁹ Whilst the mathematics of this method are relatively simple, they are based on the assumption that the plane of section passes through the nuclear equator. Selection of only nuclei that are sectioned through their centres is therefore essential for an accurate result. A number of ploidy packages now use a montage routine which displays all nuclei.^{17,18} This permits deletion of inappropriate nuclei, including those which are pale (that is, not sectioned through their centres). However, this is a time consuming and subjective procedure. A quicker, more objective method of ensuring that only correctly sectioned nuclei are selected involves the use of the transmittance/area scatter plot. A comparison of the rat imprint and section plots

gives a clear indication of the effect of sectioning the same nuclei randomly (figs 1B and 2A). If a nucleus is not sectioned through its centre there is less DNA present in the section so that its transmittance will be higher and its profile area will be less. Thus, all nuclei that are scattered to the right and down from the well defined top edge of the cluster are not sectioned through their centres. Similar scatter plots of optical density/area have also been used to help distinguish between groups of tumour cells.²⁸ An alternative method for selecting nuclei is to set the threshold so that only the darkest nuclei are selected. This is effective for smaller, darker nuclei, but considering the large size and transmittance range of nuclei which are sectioned correctly (fig 2A), selection of only the darkest nuclei may bias the data. Our results indicate that selection of all possible nuclei followed by objective exclusion using the transmittance/area plots is quicker and less biased.

The shapes of nuclei have been recognised as a complicating factor. There have been many excellent papers which have looked at methods for analysing rat hepatocytes (that is, spherical nuclei) in tissue sections.¹⁷⁻¹⁹ However, most tumour nuclei are not spherical and it has been recognised that these represent a serious problem in the analysis.¹⁹ The IOD of elongated nuclei is underestimated if it is assumed that they are spherical and if all elongate profiles are removed by filtering. The remaining circular profiles will not truly represent the volumes of each nucleus. The assumption that the nuclei are regular ellipsoids has been investigated briefly,¹⁹ but the mathematics required for this are unwieldy. In the analysis of more irregular tumour nuclei, the simple solution reported here can be seen to solve most of the mathematical challenges and produce accurate results (tables 2 and 3).

The same result was achieved with rat liver sections of different thicknesses. Any analytical system must be capable of producing the same result from the same tissue sectioned at different thicknesses. The differences in the CVs of these data are related to the section thickness and the fraction of nuclear volume within the section. A population of smaller nuclei has a greater nuclear fraction in the section and therefore a lower CV, as less mathematical extrapolation is necessary.²⁹ However, there is a limit to the use of thicker sections. Very thick sections (12 µm) have been used successfully in some applications⁸ but in most specimens there is too much nuclear overlap to make analysis practicable. For these reasons we decided to use 7 µm thick sections for further analysis. The results from the rat liver sections indicated that the computer imaging, edge and volume corrections work successfully.

There was close agreement between the results obtained with tissue sections and cytopsins of whole nuclei from breast tumours of varying grades. Preparations of whole nuclei are generally regarded as the "gold standard" in ploidy analysis and the ultimate proof of any analytical system using tissue sections is to obtain consistent results from "real" specimens. The pathology of the breast specimens analysed ranged

from epithelial hyperplasia with regular, rounded nuclei to high grade malignancies with pleomorphic nuclei. Incorporating all the above corrections (including shape correction) into one analytical system produced excellent results from sections of all of these breast specimens, which correlated well with the data from cytopsins of the same blocks.

The main advantage of DNA ploidy analysis in tissue sections lies in the ability to visualise cellular morphology, so that any associated "rare events" are not lost. As stated above, malignancy is the end result of a number of genetic insults¹ and therefore the recognition and analysis of the most genetically damaged cells holds the key to the assessment of prognosis. The analysis of invasive cells is essential for tumour prognostication and this can only be carried out effectively using tissue sections. For such analysis to be reproducible and consistent, adequate care must be given to all theoretical and technical aspects of the procedure. Only then can DNA ploidy analysis be meaningful and be considered together with other indicators to improve tumour prognosis and thus patient management.

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