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Ploidy analysis on Wilms' tumour touch imprints using ethidium bromide and automated image analysis integrated confocal laser scanning microscopy

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Abstract Although image analysis (IA) is increasingly being used to quantitate nuclear DNA, comparative data between fluorescence methods of IA and flow cytometry (FCM) is limited. In this study fluorescence IA was compared with FCM data in a series of Wilms' tumour touch preparations. Airdried touch imprints that had previously been Giemsa stained were restained with ethidium bromide. Confocal fluorescence images were obtained with a confocal laser scanning microscope and assessed by a fully automated IA package. Data was collected from 400 nuclei per imprint. The resulting DNA histograms were analysed and ploidy status and DNA indices determined using standard criteria. Results were compared with those derived from FCM analysis of nuclear suspensions. Ten of twelve tumours were concordant by both techniques. However in two cases assessed as diploid by FCM, IA identified aneuploidy. Excellent correlation between DNA indices as assessed by both techniques was observed (r=0.987). In the three cases for which both unstained and Giemsa stained touch imprints were available for IA, the histogram configurations did not differ significantly. Fluorescence IA is an accurate and sensitive technique for DNA quantitation, which appears at least comparable to FCM assessment and which has a number of important advantages.

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Introduction

Ploidy analysis provides useful prognostic information in a variety of malignant tumours [2]. DNA quantitation by flow cytometry (FCM) of nuclear suspensions is widespread. However, this technique is unselective and errors in the interpretation of DNA histograms thus obtained can occur when the presence of a minority population of aneuploid cells is masked by a preponderance within the sample of diploid cells [8, 12]. Assessment of DNA content by image analysis (IA) of nuclei stained with dyes which bind stoichiometrically to nucleic acids avoids this problem, as direct visualisation of the tumour is possible thereby allowing selection of obviously malignant cells for subsequent analysis [17, 20]. Standard methods of IA involve examination of cellular monolayers, derived from either nuclear cytospins of digested tissue blocks [8] or touch imprints [20]. The ability to assess small samples such as touch imprints and needle aspirates is an important advantage of this technique.

At present ploidy determination using IA is most commonly performed by measuring the absorbance of light following Feulgen staining [1, 4]. In general satisfactory results can be obtained, although a limited signal to noise ratio, staining variability and the need for background calibration require consideration [4, 5, 17].

Several advantages exist for using fluorescent dyes in IA. Measurement of an emitted signal, against a non-emitting background, is associated with an improved signal to noise ratio as a consequence of blur- and glare-free imaging [13]. Also fluorescent signals are easier to quantify when using modern scanning methods of confocal laser scanning microscopy and photomultiplier tube systems [14]. Although the technology to develop such systems has been available for some time, there are very few examples of DNA quantitation using fluorescence microscopy IA [11, 13, 14, 19]. Several fluorochromes

have been used for the purpose of DNA cytometric analysis including propidium iodide [11], DAPI [13, 14], Hoechst 33258 [13, 14] and chromomycin A3 [19]. At present there are no reports describing the use of ethidium bromide. This dye is a suitable fluorochrome for both FCM and IA since it may be excited with either an argon laser, a mercury lamp or a xenon lamp. It is a phenanthridinium intercalator which binds to DNA with little or no base pair preference.

In this study we quantified total nuclear DNA using an automated fluorescence IA system on touch preparations of a series of Wilms' tumours stained by ethidium bromide and compared this technique with that of flow cytometry.

Materials and methods

Study Group

Tissue samples from 12 consecutive, histologically confirmed childhood Wilms' tumours collected between 1989 and 1993 at the Department of Fetal and Infant Pathology, Liverpool were analysed.

Fluorescent Image Cytometry

Specimen preparation

Touch preparations of all cases of suspected Wilms' Tumours are routinely collected in the Department of Fetal and Infant Pathology, Liverpool. Giemsa stained, airdried touch imprint specimens had been prepared and stored in all 12 cases analysed in this study. Non-stained, airdried touch imprints were also available for 3 of the patients.

Fluorescent staining

Slides which had been previously Giemsa stained and mounted for diagnostic purposes required removal of coverslips prior to staining with ethidium bromide. This was carried out by placing the slides in xylene for a period of up to 4 days.

All slides, both unstained and previously Giemsa stained, were bathed in ethidium bromide stock solution at a concentration of 1 mg/l, prepared from powder (Sigma, UK), for a period of 3 min, at room temperature [9]. Slides were subsequently rinsed in distilled water for a few seconds and then placed in baths of successively increasing alcohol concentrations (70, 80, 90 and 100%), to cause dehydration of the cells. The slides were then placed in xylene for a few seconds, to facilitate clearing, before being mounted with 1% phenylendiamine dissolved in phosphate-buffered saline at pH 7.4 in order to prevent fluorescent bleaching [11].

Automated fluorescence image analysis system

Confocal fluorescence images were obtained with an MRC-600 confocal laser scanning instrument (Biorad, UK), connected to an Optiphot upright microscope (Nikon, Japan). The instrument was controlled by a 16 MHz 80386/87 MS/DOS Nimbus microcomputer (RM, UK) and a fully automated IA package (Fenestra Confocal Technologies, Liverpool, UK). The light source was a 25 mW multiline Argon Ion Laser (Ion Laser Technology, USA). A ×60 objective (Plan Apo 60, Nikon) of high numerical aperture (1.4), with immersion oil, was used to ensure a high confocal resolution [3]. Other systems which resemble this have been described [11, 19].

Scanning was made at ×2.0 electronic zoom so as to obtain, together with the objective, a final resolution sufficiently high for DNA measurements. For each nucleus analysed the software package gave an area (in pixels), a minimum, maximum and mean value for the intensity of the pixels and a total fluorescence signal (mean intensity × nuclear area). Each final confocal image of 256×256 pixels was obtained by averaging five image inputs (each of 1s scanning time), to reduce noise.

Ploidy analysis

Total emitted fluorescence signal per individual nucleus was assessed by setting the focal depth of the microscope at 2 μm and making manual segmentation of this from the background (including all cytoplasmic remains) using the standard internal software. Data was collected from 400 individual nuclei for each touch imprint. Nuclei were sampled by counting all those intersected by a straight line, in successive fields, across the touch imprint.

The data was processed to be accepted on line by a statistics package (ARCUS Professional, version 1.43.T) and DNA histograms were obtained. The nuclear signal distributions for each patient were recorded using a 20 class histogram technique. Analysis of a total of 400 nuclei resulted in a stable histogram configuration in all cases.

An assessment of aneuploidy was given if the histogram showed at least two distinct G0/G1 populations, each comprising at least 5% of the total sample and having a corresponding G2M population [7].

For each patient with an aneuploid histogram the DNA index was calculated as the ratio of the modal fluorescent channel number of the abnormal (aneuploid) G0/G1 peak to the modaL channel number of the normal (diploid) G0/G1 peak. In those cases in which more than one distinct aneuploid cell population was present within a single specimen's histogram (multiploid), DNA indices were calculated for each aneuploid line.

Lymphocytes identified in all tumour touch imprint specimens were sufficient in number to create an internal control as a diploid standard [6].

Flow Cytometry

Specimen preparation

Sections (30 μm thick) of formalin-fixed, paraffin-embedded tumour blocks were prepared according to the protocol described by Herman [10] using propidium iodide as the fluorescent dye. The touch imprint specimens used for fluorescence IA had been prepared from the fresh cut surface of the same blocks prior to fixation.

Flow cytometric analysis system

After preparation speciments were analysed using a Coulter Epics (R) Profile-II flow cytometer equipped with a 488-nm argon laser. Twenty thousand cells per sample were measured.

Ploidy analysis and interpretation

Data was analysed using Cytolog software. The same criteria for aneuploidy were applied as given above. Fluorescent beads were used as a diploid standard.

Results

In all 12 cases evaluable DNA histograms were obtained using both FCM and fluorescence IA. The results of DNA quantitation by the two different techniques are shown in Table 1: 10 of 12 tumours were concordant by

Table 1 The results of ploidy analysis using fluorescence image analysis and flow cytometry of 12 consecutive Wilms' tumours

Patient number	Flow Cytometry	Giemsa/ unstained	Image Analysis
1	Aneuploid	Giemsa	Aneuploid
2	Aneuploid	Giemsa	Aneuploid
3	Aneuploid	Giemsa	Aneuploid
4	Aneuploid	Giemsa	Aneuploid
		Giemsa	Aneuploid
5	Aneuploid	Unstained	Aneuploid
		Giemsa	Aneuploid
6	Aneuploid	Unstained	Aneuploid
		Giemsa	Aneuploid
7	Di/aneuploid[a]	Unstained	Diploid
		Giemsa	Diploid
8	Diploid	Giemsa	Diploid
9	Diploid	Giemsa	Diploid
10	Diploid	Giemsa	Diploid
11	Diploid	Giemsa	Aneuploid
12	Diploid	Giemsa	Aneuploid

[a] This patient's material contained both aneuploid and diploid areas as determined by flow cytometry. The touch imprints were prepared from the diploid area

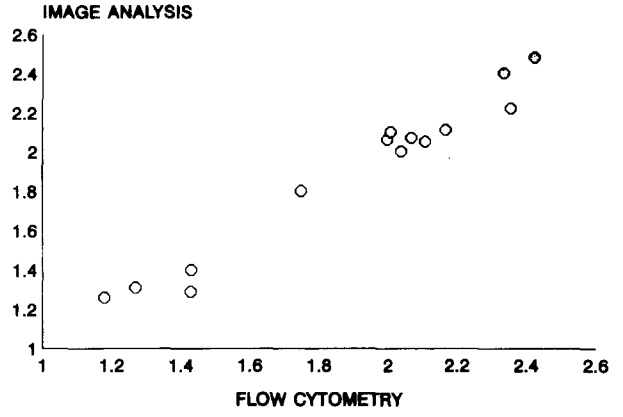

Fig. 1 Scattergram showing the relationship between the DNA index of (aneuploid) Wilms' tumours as determined by fluorescence image analysis (IA) of touch imprints (y-axis) and flow cytometry (FCM) of digested, formalin-fixed, paraffin-embedded nuclei (x-axis). Spearman's rank correlation coefficient r=0.987

FCM and IA. However in 2 cases assessed as diploid by FCM, IA identified (single) aneuploid populations.

Figure 1 shows the relationship between the DNA indices obtained for individual tumours by the two techniques (r=0.987, Spearman's rank correlation coefficient).

In the 3 cases for which both unstained and Giemsa stained touch imprints were available for IA, the histogram configurations did not differ significantly (Table 1).

Discussion

This study assessed the accuracy of our automated fluorescence image analysis system when compared with flow cytometry by measuring the DNA content of 12 consecutive Wilms' tumours. All 6 tumours previously assessed as

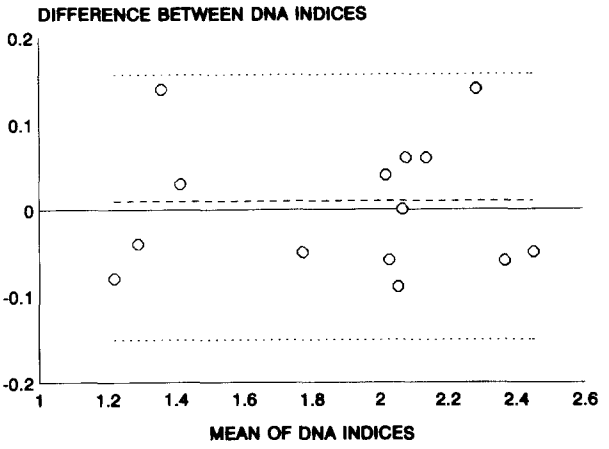

Fig. 2 The difference between the DNA indices as determined by fluorescence IA and FCM (y-axis) versus the mean DNA index by both techniques for individual (aneuploid) Wilms' tumours. In those cases in which multiple aneuploid populations were identified within a single tumour, the DNA index for each are plotted separately. The mean of the difference between the DNA indices by the two techniques together with the 95% prediction intervals are plotted as horizontal lines. Note that the prediction intervals span the abscis indicating no significant difference between the DNA index as assessed by fluorescence IA or FCM.

aneuploid by FCM were confirmed as such by IA. In the 6 remaining patients, IA detected aneuploid populations in 2 tumours classified as diploid by FCM (Table 1). Fluorescence IA thus appears a robust and sensitive technique for ploidy determination on touch preparations.

Similar comparative observations have been reported in normal adult rat liver and in situ carcinoma of the human oesophagus [13, 19]. The increased sensitivity of IA at detecting aneuploid populations has been commented on by others: in an analysis of bladder washing cytospins from patients with a history of bladder tumours, IA provided information of greater clinical significance than FCM [12].

There is an excellent correlation between DNA indices as determined by fluorescence IA and FCM (r=0.987). Use of the correlation coefficient may, though, be misleading since this parameter merely reflects straightness of the scattergram line (Fig. 1) rather than any numerical similarity between respective DNA index values. However the remarkable similarity between numerical data obtained by the two techniques is apparent from Figure 2, in which absolute differences between paired sets of DNA indices are plotted against their means. There is no significant difference between IA and FCM, indeed the maximum predicted difference for any particular lesion between the DNA index as assessed by the two techniques is <0.2. It must be stressed that, in addition to binding DNA, both ethidium and propidium intercalate between the bases in the relatively small amounts of double stranded RNA also present. Thus when assessing differences in nuclear DNA content per se between individual specimens, pre-treatment with RNase is mandatory. However for the purpose of this comparative study such treatment was considered non-essential.

In addition to probable increased sensitivity for the detection of aneuploidy and its applicability to small tissue samples, fluorescence IA offers other advantages over FCM. The preparation of Giemsa stained cellular monolayers such as touch imprints is a routine activity in many laboratories. This technique thus offers an assessment of ploidy status which is both non-destructive to the tissue analysed and which may be performed retrospectively. Ethidium bromide can also be removed from nuclei using a suitable solvent, allowing for subsequent additional investigations [16]. Recently automated fluorescence IA has been used to analyse multiple parameters including specific antigens and nucleic acid sequences, to localise these molecules within the cell and to study the spacial relationships between distinct molecular species and between individual cells [15].

Several authors have endeavoured to quantify DNA in histological sections of tissue blocks with fluorescence IA. However the fact that the whole of a nucleus (typical diameter approximately 7–8 μm) cannot be contained within the optical section of the confocal microscope (maximum 3.5 μm) has proved a problem (this is not the case with touch imprints in which airdrying results in the nuclear height being less than the optical section thickness and thus the whole of the DNA emission signal for a particular nucleus being included in a single scan) [5]. However preliminary data suggests that this may not be insurmountable [18, 19]. Certainly the ability to marry accurate ploidy information with preservation of tissue architecture, and thus for example localise differences in ploidy to individual histological components of a tumour visualised in a section, would be invaluable.

Resistance to IA is often based upon its perceived greater cost both in money and time. However, after the initial outlay for the confocal microscope (approximately £100,00 – presently decreasing – and comparable to that of a flow cytometer) costs are only attributed to reagents, electricity and technician's time. Duration of analysis is approximately 2 h, but with refinements in software this will decrease.

In conclusion, fluorescence image analysis is an accurate, sensitive technique for DNA quantitation, which appears at least comparable to flow cytometric assessment and which has a number of important advantages. We believe that further assessment of this technique is warranted.

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