Pergamon

European Journal of Cancer, Vol. 35, No. 5, pp. 787–795, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00427-4

Original Paper

Reliability and Significance of DNA Measurements in Interphase Nuclei and Division Figures in Histological Sections

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DNA contents from single cells at interphase and division were analysed in histological sections and in imprints from 73 breast cancer specimens. Fetal livers from 18 terminations of normal pregnancies provided the standard for truly mitotic prophases, metaphases and telophases. The reliability of DNA quantities from image microphotometry was improved using paraffin-embedded tissue samples from which 4, 8 and 15 μm slices were Feulgen stained. Imprinted replicas from the mirror surface of each freshly cut specimen provided matching domains and represent the crucial approach in this project. A close positive relationship was observed between interphase nuclei in 8 μm sections and their imprinted counterparts ($r = 0.992$; $n = 73$). Interphase nuclei in 4 μm sections yielded insufficient DNA contents when compared with the imprints ($r = 0.815$; $n = 21$) and with endogenous lymphocyte nuclei. This 2 c DNA standard also calibrated 232 mitotic figures to 3.91 ± 0.01 c in 15 μm sections from fetal liver. Prophases, metaphases and telophases were slightly scattered (coefficient of variation = 0.04 each). The 0.09 c deficiency to plain 4.0 c was read as an artifact from sectioning. However, the methodical bias did not challenge the most irregular DNA distribution profiles recorded from chromosome division figures (CDFs) in 15 μm sections of breast cancers. Poorly differentiated and aggressive breast cancer (Auer type IV, Zetterberg type A) exhibited a 4.5 c exceeding rate of 82.24% from a total of 752 CDFs in 10 randomly selected cases. Well differentiated, slowly growing cancer with diploid interphase nuclei (Auer I, Zetterberg D) surprisingly showed a 4.5 c exceeding rate of 29.26% from a total of 173 mitoses and CDFs in 10 randomly selected cases. The bulk of data beyond the mitotic 4.0 c level discriminates biological bias from methodical impairment. We concluded that 8 μm sections are sufficient for human interphase nuclei, whereas a depth of 15 μm preserves intact mitoses and CDFs. © **1999 Published by Elsevier Science Ltd. All rights reserved.**

Key words: breast cancer, chromosome division figure, microphotometry, mitotic DNA standard
Eur J Cancer, Vol. 35, No. 5, pp. 787–795, 1999

INTRODUCTION

HISTOLOGICAL MICROSECTIONS represent classical specimens in routine pathology. From microslices, the thesaurus of pathological experience has been established. Electron microscopy, enzyme histochemistry, cytogenetics, immunohistochemistry and molecular biology have contributed valuable progress. An understanding and interpretation of pathological data still demand the morphological background of sections.

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Received 28 Jul. 1998; revised and accepted 4 Nov. 1998.

Study of the DNA content of the nucleus has been inspired by the famous technique of ultraviolet microphotometry [1]. Currently, measurement in visible light does not require such an expensive set-up and is a simple and quick procedure. The method demands the specific staining of DNA [2–4], and the Feulgen technique is certainly one of the most reliable techniques of histochemistry [5].

Since the development of Feulgen staining, many publications have dealt with the microphotometry of cell nuclei using microslices [6–14]. Uncertainties remain regarding the danger of overlapping and cut nuclei. To overcome these systematic errors, methods of correcting DNA measurements in tissue sections have been suggested [10, 15, 16].

The detection of asymmetric divisions of cell nuclei in cancers is associated with a remarkable observation of Hansemann in 1891: "The sections must not be too thin, otherwise many cells would become cut through" [17]. The present paper deals with this suggestion. We systematically investigated the impact of different section thicknesses on the reliability of microphotometric data. Cell nuclei were measured in interphase and during mitosis. Prophases, metaphases and telophases from breast cancer, although appearing to be normal in the light microscope, were identified by their DNA amounts as pathological entities. This result confirmed the chromosome division figures (CDFs) defined previously [18].

MATERIALS AND METHODS

Samples

The material comprised 73 cases of invasive breast carcinoma. Additionally, 18 terminations of normal pregnancies provided the standard for mitotic divisions. The tumours and terminations were from patients of the Flensburg district, northern Germany, and the tumours were originally diagnosed at the Institute of Pathology, Flensburg. For comparative investigation, the tumours were selected from a 2-year period of consecutive cases on the basis of their DNA histogram types, first analysed with ACAS equipment (Ahrens, Bargteheide, Germany).

Sections and imprints

Initially, breast tumours were carefully prepared, connective tissue was removed, and the freshly cut tumour surface imprinted to four microscope slides. The four air-dried imprints were fixed in formalin. The surface of half the tumour was diagnosed in a frozen section and was proven to contain only tumour cells. The other half of the specimen was fixed in buffered 4% formaldehyde, paraffin-embedded and stored.

Feulgen staining

The cell and tissue preparations were rehydrated in decreasing ethanol concentrations and exposed to hydrolysis in one batch of 5 M HCl at 22°C for 60 min. The specimens were then rinsed in distilled water and stained with one batch of Schiff's reagent at room temperature for 90 min. The samples were washed three times in sulphurous acid (10 ml $Na_2S_2O_5$, 10 ml HCl, 180 ml distilled water). Finally, they were rinsed under running tap water, dehydrated in increasing ethanol concentrations, transferred to xylene and mounted in Eukitt (refractory index 1.494).

Investigation progress

For comparative studies, the Feulgen stained imprints were measured once more with a high resolution instrument (prototype, Karolinska Institute, Stockholm, Sweden). The second halves of the paraffin-embedded tissues (73 cases) were carefully adjusted in the microtome and cut in 8 ± 1 μm sections. Our aim was to produce sections which exactly match their corresponding imprints. Additionally, 4 ± 1 μm sections were made from the identical material of 21 specially selected cases. Subsequently, fetal livers from terminations were sectioned in 15 μm slices and measured for the 4 c DNA standard of mitotic prophases, metaphases and telophases after Feulgen staining. Similarly, 15 μm slices were cut from prediagnosed and premeasured breast cancers in order to evaluate CDFs. This was carried out with 10 randomly selected cancers with diploid and 10 cancers with aneuploid DNA distribution profiles of interphase nuclei.

Measuring procedure

The DNA content of individual cell nuclei was measured at random within the selected area. In each case, 150 interphase nuclei of tumour cells were measured. Four important advantages of optical control by an operator highly experienced in morphology improved data reliability:

(1) the correct tumour area, corresponding with the diagnosis, has to be selected for measurement on the basis of morphological criteria;
(2) tumour cells must be identified correctly in sections as well as in imprints;
(3) overlapping nuclei and those with too close a contact or with degenerated nuclear membranes must be precluded;
(4) exclusion is especially required for nuclei which were cut during microsectioning. This was achieved by focusing each nucleus up and down, searching for gaps (spots without contrast) on the screen monitor. In each case, at least 20 small lymphocyte nuclei were assessed using their mean as an internal standard for the diploid (2 c) DNA content.

Nuclear divisions were morphologically identified as prophases, metaphases or telophases; they were identified as CDFs by their aberrant DNA content. Multipolar figures or apoptotic entities were intentionally precluded from microphotometry. All specimens were measured by the same operator using only computer assisted image analysis systems, either the ACAS system at Flensburg [19] or the Karolinska prototype. The latter was based on an axioscope (Zeiss), equipped with an immersion plan objective (40/0.95; Nikon) and a CCD camera (Nikon).

Histogram interpretation [20]

Using image microphotometry of sections and imprints, histograms of DNA contents were obtained from interphase nuclei which were interpreted according to a modified subjective method. The original method was used for the classification of DNA profiles obtained in fine-needle aspiration specimens from breast tumours. Histograms characterised by a single peak in the 'diploid' or 'near-diploid' region (1.5–2.5 c) were classified as type I. The total number of cells with DNA values exceeding the 'diploid' region (> 2.5 c) was $< 10\%$. Type II showed a single peak in the 'tetraploid' region (3.5–4.5 c) or peaks in both the 'diploid' and the 'tetraploid' regions ($> 90\%$ of the total cell population). The number of cells with DNA values between the 'diploid' and the 'tetraploid' regions and those exceeding the 'tetraploid' region (> 4.5 c) was $< 10\%$. Type III histograms were characterised by DNA values ranging between the 'diploid' and the 'tetraploid' regions. Only a few cells ($< 5\%$) had more than 4.5 c. This distribution type of DNA content is difficult to interpret and might comprise both highly proliferating 'near-diploid' and aneuploid populations. Type IV showed increased ($> 5\%$) and/or distinctly scattered DNA values exceeding the 'tetraploid' region (> 4.5 c). These histograms were suggested to reflect 'aneuploid' populations of interphase nuclei with decreased genomic stability.

Histogram interpretation [21]

The method [21] was originally used for the primary diagnoses of prostatic carcinomas of 3874 patients and was based on the prognostic impact of their follow-up. The tumour cell populations were calculated in the form of 'window typing': D type, more than 50% of the measured interphase nuclei are in the diploid region or the tetraploid region. The modal value falls into the diploid region; T type, more than 50% of the measured nuclei are in the diploid or the tetraploid region. However, the modal value is in the tetraploid region; A type, more than 50% of the nuclei possess DNA contents beyond the diploid and tetraploid regions.

RESULTS

Interphase nuclei

DNA measurements from 73 cases of breast cancer are given in Table 1, comparing the mean ploidy of the main nuclear fraction, the 2.5 c exceeding rate (ER) and the 5 c ER from imprints and 8 μm sections, respectively. Each case was diagnosed histologically and, after measuring a sample of 150 nuclei, was classified by the distribution profile of its nuclear DNA contents.

Accuracy of measurements was expressed with the linear correlation of nuclear DNA from imprints and 8 μm sections replica, $r = 0.992$. The linear regression was demonstrated with $y = 0.967x$ (Figure 1). With regard to the ploidy value, the mean difference between the imprints and their corresponding 8 μm sections was 0.095 ± 0.016 c where scattering is given as the standard error of the mean (SEM). The mean 2.5 c ER was $50.19 \pm 4.95\%$ from imprints and $47.94 \pm 5.12\%$ from 8 μm sections, whereas the 5 c ER averaged $11.69 \pm 2.41\%$ from imprints and $9.55 \pm 2.31\%$ from 8 μm sections. In other words, representative samples of cancer cells were evaluated both in imprints and 8 μm sections. The absolute differences from the 2.5 c ER and the 5.0 c ER averaged $5.50 \pm 0.94\%$ and $4.24 \pm 0.92\%$, respectively (each $n = 73$). The mean difference could be balanced by 2 nuclei/sample. Data scattering from 2 c to 4 c and beyond 5 c DNA is caused by the biological bias of breast cancer cells (Table 1, Figure 1).

In a smaller sample of 21 selected cases the imprints were compared with 8 μm and 4 μm sections (Table 2). The depth of 4 μm appeared to be inappropriate for reliable DNA content of human interphase nuclei, when compared with the imprinted counterparts ($r = 0.815$) and with the endogenous lymphocyte nuclei. The DNA deficit from 4 μm sections yielded a regression with $y = 0.355x$. In contrast, the corresponding 8 μm sections yielded a linear regression $y = 0.958x$ (Figure 2), similar to the complete sample (Figure 1).

Case nos. 8, 14, 63 and 68 were chosen to illustrate the technical bias caused by insufficient thickness (Figure 3). Most affected were specimen nos. 63 and 68 with aneuploid distribution profiles from their imprinted interphase nuclei. The deficit from injured nuclei in the 4 μm section produced a rather diploid profile (Figure 3), but, the profiles from 8 μm sections followed those of the imprints with high fidelity and good resolution. This general finding is illustrated for case nos. 65 and 70 showing DNA aneuploidy (Figure 4).

Errors in DNA content from inappropriate specimens can scarcely be estimated by nuclear morphology in the microscope, especially in diploid cases (Figure 5a, b). The technical bias from insufficient depth becomes obvious only if microphotographs are compared. In particular, the aneuploid nuclear population had less optical density in the 4 μm sections (Figure 5c) than in the 8 μm sections (Figure 5d). To demonstrate further the impairment by insufficient depth, the dimensions of 4 μm and 8 μm sections were projected on microphotographs. The 4 μm section hardly covers complete interphase nuclei of a diploid population (Figure 6a) and frequently cuts nuclei of an aneuploid sample (Figure 6b), whereas a depth of 8 μm is sufficient for both types. Thus, the systematic error caused by the smaller depth immediately becomes evident.

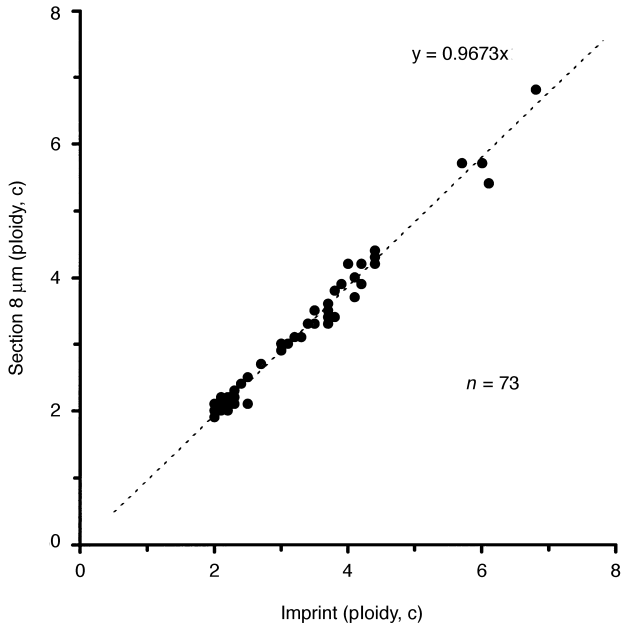

Figure 1. Reliable amounts of nuclear DNA in 8 μm sections relative to replica imprints. The sample ploidy of 150 interphase nuclei was recorded in each of 73 cases of breast cancer (cf. Table 1).

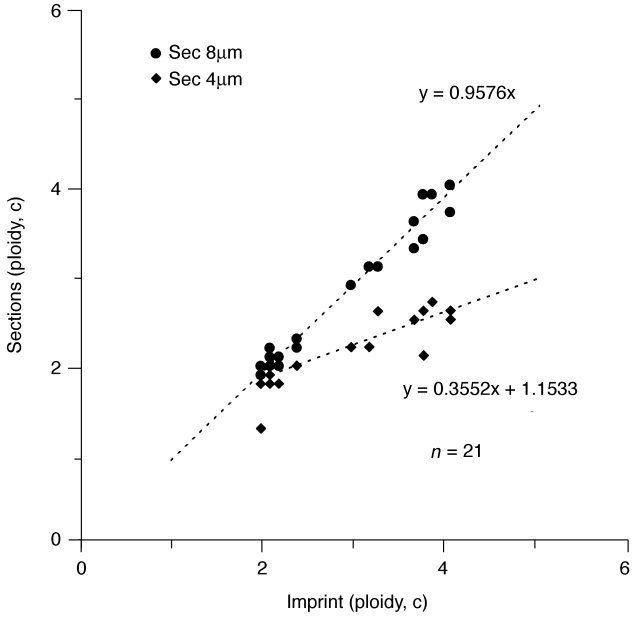

Figure 2. Quantitative comparison of DNA records from interphase nuclei in 8 and 4 μm sections. The sample ploidy of 150 interphase nuclei was recorded from each of 21 randomly selected breast cancers. Larger nuclei (> 2 c DNA) show poor correlation with the replica imprints.

Table 1. *Microphotometric comparison of imprints and microsections*

Case no.	Histology diagnosis	Imprint					8 μm section				
		A	Z	Ploidy	> 2.5 c	> 5.0 c	A	Z	Ploidy	> 2.5 c	> 5.0 c
1	A	I	A/D	2.3	13.0	0.0	I	D	2.1	7.3	0.0
2*	A	I	D	2.1	10.0	2.7	I	D	2.1	7.3	0.0
3	C	I	–	2.1	0.0	0.0	I	D	2.1	0.0	0.0
4*	A	I	D	2.1	12.0	0.7	I	D	2.0	11.3	0.0
5	G	I	D	2.2	8.7	0.0	I	D	2.1	10.0	0.0
6	A	I	D	2.1	5.3	0.0	I	D	2.1	5.3	0.0
7	A	I	D	2.2	3.3	0.0	I	D	2.1	2.7	0.0
8*	A	I	D	2.0	18.0	1.3	I	D	2.0	8.7	0.0
9	E	I	D	2.1	9.3	0.0	I	D	2.1	2.7	0.0
10	B	I	D	2.1	6.7	0.7	I	D	2.1	4.7	0.7
11*	F	I	D	2.1	2.7	0.0	I	D	2.0	0.7	0.0
12*	B	I	D	2.0	24.7	2.7	I	D	2.0	16.7	1.3
13	B	I	D	2.1	12.0	0.0	**II**	D	2.1	5.3	0.0
14	B	I	D	2.0	14.0	0.0	I	D	1.9	4.0	0.0
15	A	I	D	2.0	15.3	0.7	I	D	2.0	12.7	0.0
16	C	I	D	2.2	10.7	1.3	I	D	2.2	12.8	0.0
17	A	I	D	2.1	6.7	0.0	I	D	2.1	3.3	0.0
18	C	I	D	2.0	4.7	0.0	I	D	2.0	6.7	0.0
19*	A	I	D	2.0	8.7	0.7	I	D	2.0	9.3	0.7
20*	A	I	D	2.1	4.7	0.0	I	D	2.1	2.0	0.0
21	B	I	A/D	2.5	5.2	2.7	I	A/D	2.2	4.7	0.0
22	B	I	D	2.1	6.0	0.0	I	D	2.1	8.7	0.0
23	A	I	D	2.1	13.3	1.3	I	D	2.1	4.7	0.0
24	A	III	D	2.1	30.7	0.7	III	D	2.2	22.0	0.0
25	A	III	A/D	2.4	42.0	1.3	**I**	D	2.2	10.7	0.0
26	A	III	A/D	2.4	47.3	0.0	**I**	A/D	2.3	13.3	0.0
27	B	III	A/D	2.4	57.3	3.3	III	A/D	2.4	29.3	0.0
28	G	I	D	2.1	2.0	0.0	I	A/D	2.2	15.3	0.0
29	A	I	A/D	2.3	19.3	1.3	I	D	2.2	5.3	0.0
30	B	I	D	2.2	4.7	0.0	I	A/D	2.3	26.0	0.0
31	A	I	D	2.2	10.0	0.7	**III**	A/D	2.5	44.7	0.7
32	A	I	D	2.0	11.3	0.7	I	D	2.1	2.7	0.0
33	A	I	D	2.1	10.0	0.0	I	A/D	2.2	2.0	0.0
34*	A	I	A/D	2.2	13.3	0.7	I	D	2.0	12.0	0.0
35	A	I	D	2.1	3.3	1.3	I	D	2.0	0.7	0.0
36	A	III	D	2.1	24.7	0.0	**I**	D	2.2	11.3	0.0
37	A	I	D	2.1	8.7	0.0	**III**	D	2.1	15.3	0.7
38*	A	I	D	2.1	2.0	0.0	I	D	2.2	5.3	0.0
39*	A	I	D	2.2	4.7	0.0	I	A/D	2.3	3.3	0.0
40†	A	IV	D	2.2	56.0	6.0	IV	A/D	2.1	59.3	8.7
41	A	II	T	4.0	100.0	8.0	II	T	4.2	100.0	9.3
42	A	II	T	3.8	100.0	9.0	II	T	3.9	100.0	1.3
43	D	IV	D	2.2	68.0	10.7	IV	A/D	2.2	56.7	9.5
44	B	IV	T	3.5	96.7	24.0	IV	A	3.5	100.0	9.3
45	A + B	II	T	3.8	98.0	4.0	II	T	3.8	100.0	4.0
46	A	III	D	2.0	38.0	4.7	III	D	1.9	28.0	0.7
47	B	IV	T	4.1	99.3	2.7	**II**	T	4.0	100.0	4.0
48	A	II	T	4.4	100.0	14.0	II	T	4.2	100.0	7.3
49	A	IV	T	3.7	100.0	8.0	IV	A	3.4	100.0	4.0
50	A	IV	A	3.5	100.0	16.0	IV	A	3.3	99.3	9.3
51†	A	IV	T	3.9	99.3	16.7	IV	T	3.9	100.0	5.3
52	B	IV	T	3.7	100.0	12.0	IV	A	3.6	100.0	9.3
53	F	IV	T	3.7	88.0	8.0	IV	A	3.4	100.0	10.7
54†	A	IV	A	3.1	100.0	35.3	IV	A	3.0	100.0	18.7
55	A	IV	A	3.0	99.3	27.3	IV	A	3.0	100.0	11.3
56†	A	IV	A	6.8	100.0	90.7	IV	A	6.8	100.0	84.7
57	B	II	T	3.7	100.0	12.7	II	A	3.5	99.3	4.7
58	A	IV	T	4.1	100.0	20.7	IV	A	3.7	100.0	24.7
59	A	IV	A	3.3	100.0	27.3	IV	A	3.1	100.0	6.7
60	C	IV	T	4.2	77.3	5.3	IV	T	4.2	71.3	4.0
61	A	II	D	2.3	47.3	1.3	II	D	2.2	24.7	0.7
62†	A	IV	A	6.0	100.0	28.7	IV	A	5.7	100.0	83.3
63	A	IV	T	3.8	97.3	5.3	IV	A	3.4	100.0	7.3

(continued)

Table 1 *continued*

Case no.	Histology diagnosis	Imprint					8 μm section				
		A	Z	Ploidy	> 2.5 c	> 5.0 c	A	Z	Ploidy	> 2.5 c	> 5.0 c
64†	A	IV	A	6.1	100.0	98.7	IV	A	5.4	100.0	80.0
65	A	IV	A	3.4	98.7	13.3	IV	A	3.3	100.0	12.7
66	A	IV	A	4.4	100.0	22.0	IV	T	4.3	100.0	24.7
67†	A	IV	A	5.7	100.0	90.7	IV	A	5.7	100.0	84.0
68	A	IV	A	3.0	97.3	51.3	IV	A	2.9	100.0	34.0
69†	C	IV	T	3.8	98.7	18.0	IV	A	3.4	100.0	8.0
70	A	IV	A	3.2	100.0	50.0	IV	A	3.1	100.0	41.3
71†	A	IV	A	4.4	100.0	28.0	IV	A	4.4	100.0	32.7
72	A + B	IV	T	3.7	100.0	27.3	IV	A	3.3	100.0	14.7
73†	A + B	IV	A	2.7	98.0	30.7	IV	A	2.7	90.0	22.0

Histological diagnosis of breast carcinomas: A, duct; B, lobular; C, mucinous; D, medullary; E, papillary; F, tubular; G, adenoid cystic carcinoma. Cases selected for chromosome division figure (CDF) measurements were labelled with * when well differentiated, and labelled with † when poorly differentiated. Cells in bold mark differences between imprints and sections (column A). Column A, Auer's typing; column Z, Zetterberg's typing.

The interpretation and classification of histograms was carried out by two of the authors. In general, the results from the imprints and sections corresponded very well, especially in the diploid tumour group (Auer type I, Zetterberg type D). Agreement was 90.4% (66/73) by Auer's classification and 69.9% (51/73) by Zetterberg's classification. The differences were caused mainly by aneuploid cases.

Nuclear divisions

Chromosomes subject to metaphase arrangement, anaphase movement and telophase separation probably require more space than interphase nuclei. An appropriate depth of 15 μm was found with preliminary experiments.

The mitotic standard was established with fetal liver measuring 108 prophases and 101 metaphases (Figure 7a). The prophases yielded a relatively symmetrical peak at 4.0 c DNA, calibrated from small endogenous lymphocyte nuclei. The metaphase population was skewed slightly to the left, probably due to knife artifacts. A similar effect was recorded from corresponding telophase halves (Figure 7b). The complete fetal liver sample of 232 mitoses scattered only slightly (coefficient of variation (CV) = 0.04)) and averaged 3.91 ± 0.01 c, close to the expected 4.0 c value. The observed deficiency of 0.09 c demonstrates unavoidable losses due to sectioning. The 4.5 c ER of these mitoses was zero.

The depth of 15 μm was proven with CDFs from breast cancers with aneuploid and diploid distribution profiles of interphase nuclei. The 752 CDFs from 10 randomly selected cases of the aneuploid cancer type (cases marked with † in Table 1) showed 82.24 ± 3.37% 4.5 c ER and 68.21 ± 5.37% 5 c ER. The 1500 correlated interphase nuclei showed 44.35 ± 11.04% 5 c ER. The 5 c ER of CDFs surpasses that

Table 2. *Comparison of* 4 *and* 8 *μm sections*

Case no.	Imprint			8 μm section			4 μm section		
	Ploidy	> 2.5 c	> 5.0 c	Ploidy	> 2.5 c	> 5.0 c	Ploidy	> 2.5 c	> 5.0 c
7	2.2	3.3	0.0	2.1	2.7	0.0	1.8	1.3	0.0
8	2.0	18.0	1.3	2.0	8.7	0.0	1.3	1.3	0.0
14	2.0	14.0	0.0	1.9	4.0	0.0	1.9	2.7	0.0
25	2.4	42.0	1.3	2.2	10.7	0.0	2.3	6.0	0.7
26	2.4	47.3	0.0	2.3	13.3	0.0	2.0	2.0	0.0
28	2.1	2.0	0.0	2.2	15.3	0.0	1.8	1.3	0.0
34	2.2	13.3	0.7	2.0	12.0	0.0	2.1	4.7	0.0
35	2.1	3.3	1.3	2.0	0.7	0.0	2.2	2.7	0.0
36	2.1	24.7	0.0	2.2	11.3	0.0	2.0	5.3	0.0
37	2.1	8.7	0.0	2.1	15.3	0.7	1.9	2.7	0.0
42	3.8	100.0	9.0	3.9	100.0	1.3	2.6	62.0	0.7
46	2.0	38.0	4.7	1.9	28.0	0.7	1.8	16.0	0.0
47	4.1	99.3	2.7	4.0	100.0	4.0	2.5	52.0	0.7
51	3.9	99.3	16.7	3.9	100.0	5.3	2.7	74.7	0.0
52	3.7	100.0	12.0	3.6	100.0	9.3	2.5	50.0	0.7
58	4.1	100.0	20.7	3.7	100.0	24.7	2.6	70.7	3.0
59	3.3	100.0	27.3	3.1	100.0	6.7	2.6	70.0	3.0
63	3.8	97.3	5.3	3.4	100.0	7.3	2.1	20.0	0.0
68	3.0	97.3	51.3	2.9	100.0	34.0	2.2	62.0	3.0
70	3.2	100.0	50.0	3.1	100.0	41.3	2.2	58.0	6.7
72	3.7	100.0	27.3	3.3	100.0	14.7	2.5	70.0	5.3

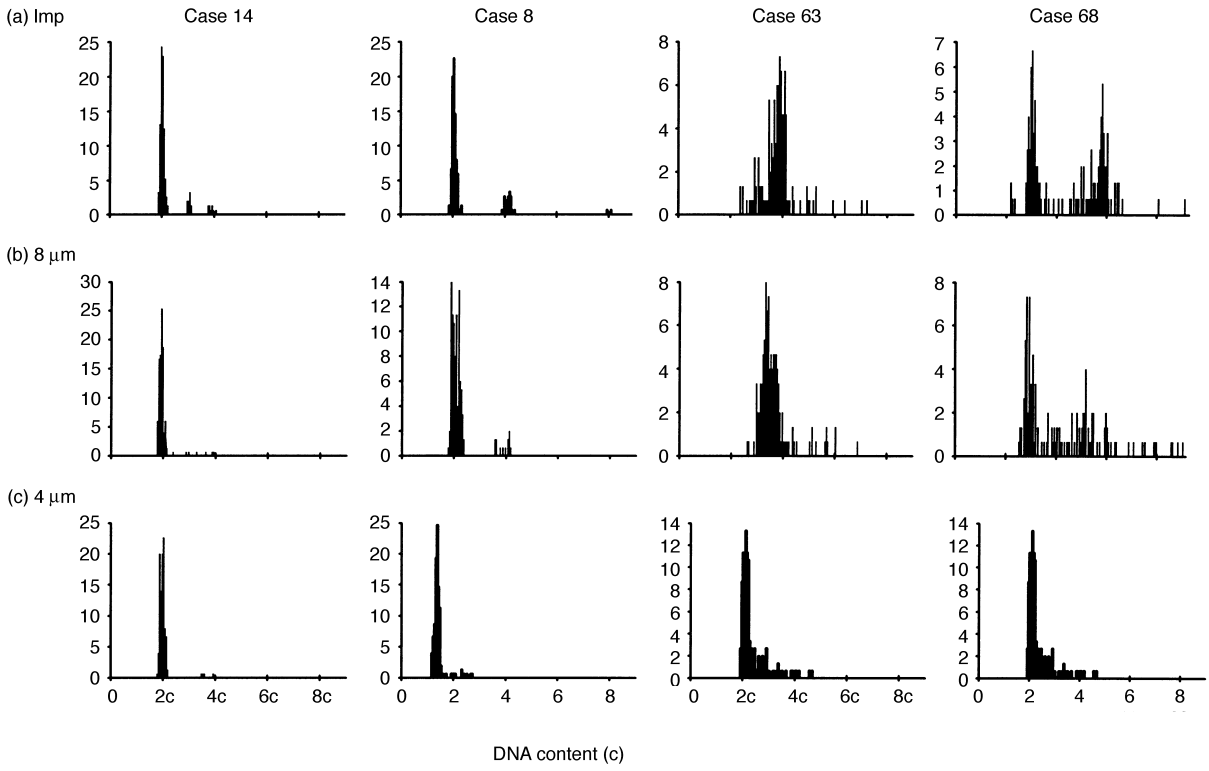

Figure 3. Representative histograms from (c) 4 and (b) 8 μm sections compared with (a) their replica imprints from breast cancers. Each DNA profile was recorded from 150 interphase nuclei. From 4 μm sections, erroneous DNA hypoploidy might be deduced in case nos. 8 and 14, whilst DNA aneuploidy might not be detected in case nos. 63 and 68.

of the interphase nuclei by a factor of 1.5. This is a remarkable kinetic parameter. In contrast, the 173 mitotic figures and CDFs from 10 randomly selected cases of the diploid cancer type (cases marked with * in Table 1) showed 29.26 ± 5.83% 4.5 c ER and 12.25 ± 3.98% 5 c ER. The 1500 correlated interphase nuclei showed only 0.88 ± 0.33% 5 c ER. Most interestingly, the 5 c ER of CDFs surpasses that of the interphase nuclei by a factor of 15. This demonstrates that many divisions escaped mitotic regulation. However, genomic disorder contributes to the quantitative phenotype of CDFs in the aneuploid, poorly differentiated type of breast cancer (Figure 8) to a much greater extent.

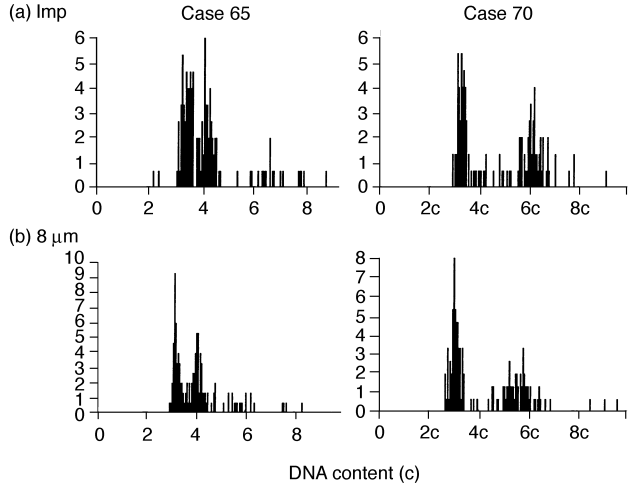

Figure 4. Microphotometric analysis of aneuploid type breast cancers. The high resolution instrument discriminated two main peaks not only in the imprint (a), but also in the 8 μm section (b) (case no. 65). Some underestimation due to cut nuclei was observed in breast cancer case no. 70.

DISCUSSION

All scientific methods using instruments and reagents are materially limited and thus prone to error. Important for the results is the accuracy and precision which result from an optimised cascade of compromises. A prerequisite for both is the specificity of diagnosis and of tissue preparation. Preventing the loss of specific material is likewise important. The accuracy of determining nuclear DNA amounts from histological slices depends on the appropriate thickness. Cut

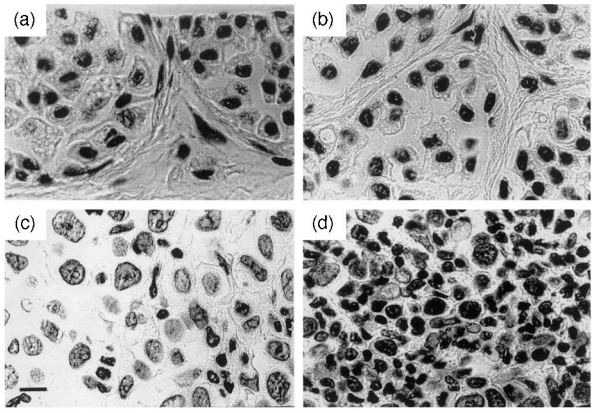

Figure 5. Effects of 4 μm (a, c) and 8 μm (b, d) depth in diploid type (a, b) and aneuploid type (c, d) breast cancer. Minor optical density and fragments (c) indicate frequently cut nuclei. Feulgen stained. Bar represents 20 μm (a–d).

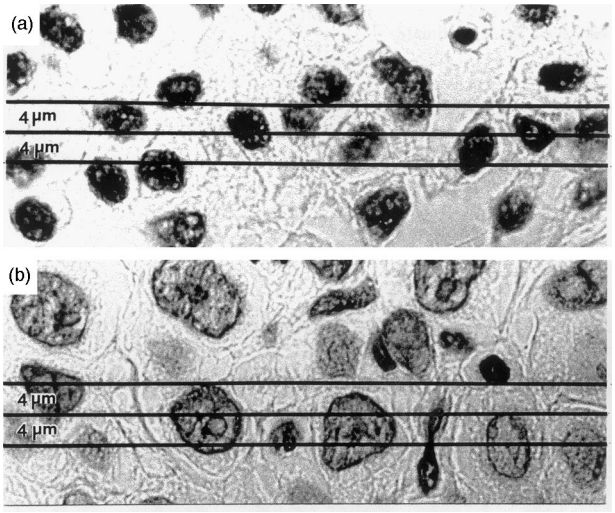

Figure 6. Demonstration of depth in microsections from breast cancer. Whilst 4 µm sections cover most interphase nuclei in the diploid type (a), 8 µm are required to cover larger nuclei in the aneuploid type (b). Feulgen stained. Bar represents 20 µm.

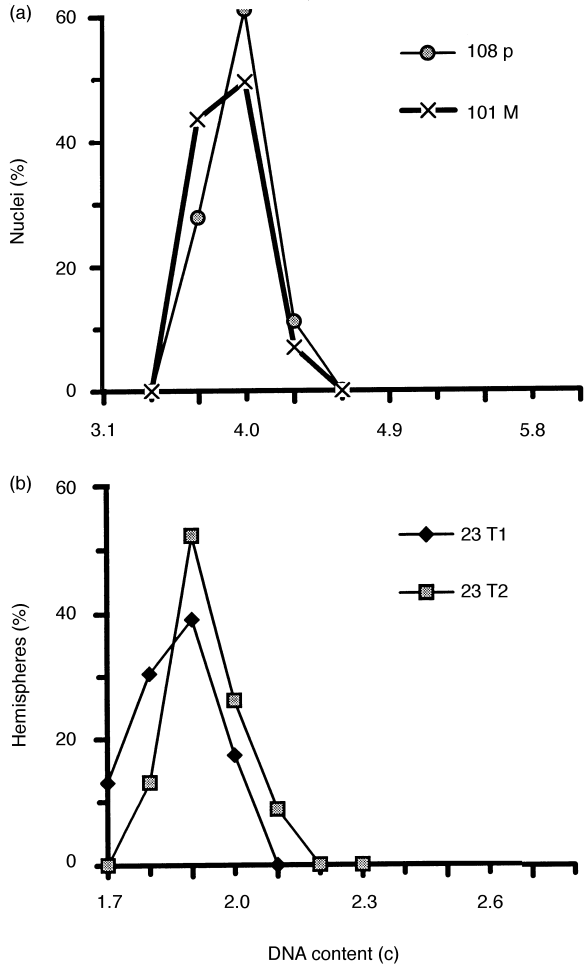

Figure 7. Mitotic standard of nuclear DNA from 18 fetal livers. Prophases (p) and metaphases (M) were recorded with peaks at 4.0 c in 15 µm sections. The deviations from plain 2.0 c DNA content in telophase hemispheres (T1, T2) are inevitable microtome artifacts.

nuclei, as well as overlapping nuclei, must be avoided. Working in visible light demands specific, stoichiometric and reproducible staining. A main factor for accuracy in microphotometry is the choice of the nuclear DNA standard to calibrate correctly sample nuclei. In the investigations described here, the interphase nuclei of small endogenous lymphocytes provide the internal 2 c standard. Mitoses from fetal livers served as a secondary standard for 4 c from mitotic prophases and metaphases, and 2 c from telophase halves. An operator contributes the subjective momentum that may guarantee or diminish specificity in diagnosis and the random selection of actual records.

Small differences in the 2.5 c ER and the 5 c ER between imprints and 8 µm sections reflect high accuracy in the random selection of nuclei at interphase from the respective samples. Linear regression and a correlation of $r = 0.992$ ($n = 73$ cases) showed that 8 µm section are sufficient for the larger nuclei as well. From microsections, the mean 2.5 c ER and the mean 5 c ER were slightly smaller than those from imprints. This would indicate a minor accuracy caused by the microtome, if a preferential imprint of larger nuclei is not assumed. Thus, the interphase nuclei are endangered by knife artifacts in microsections, whilst the imprints might provide less accuracy due to selective transfer of larger nuclei which themselves are endangered by squashing.

The dramatic loss of accuracy was demonstrated with 4 µm sections compared with 8 µm sections and relative to the imprint data (Table 2, Figure 2). Insufficient DNA amounts from cut interphase nuclei were obvious from a correlation of $r = 0.815$ ($n = 21$ cases) and the fact that the linear regression $y = 0.355x$ does not arise from the origin. Examples were chosen in order to show that DNA distribution profiles from interphase nuclei in 4 µm sections could be easily misinterpreted (case nos. 63 and 68 in Figure 3). In contrast, examples of sufficient accuracy are given with 8 µm thickness (Figure 4). Case no. 65 discriminated two separated stemlines between 3 c and 4 c not only from the imprint, but also from the section.

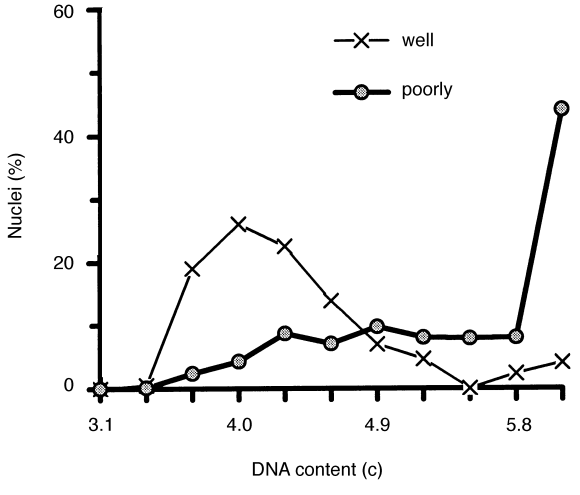

Figure 8. DNA profiles from divisions from two types of breast cancer (10 cases each). The histologically well differentiated type with diploid interphase nuclei (cases marked with * in Table 1) exhibited 173 divisions, comprising mitoses at 4.0 c and some chromosome division figures (CDFs). The poorly differentiated type (cases marked with † in Table 1) comprised 752 divisions, which were mainly CDFs with a prominent 4.5 c exceeding rate (ER).

Figure 5 provides a view through the light microscope on sectioned material. The effect of cut nuclei becomes obvious only when complete nuclei are immediately presented as an optical standard. Figure 6 clearly shows the dramatic difference between 4 and 8 μm thicknesses. The latter sufficiently covers larger nuclei present in breast cancer. Microsectioning of organic material is not a rigorous process; it allows slightly protruding nuclei to be depressed by the knife [14].

The reliability of measurements depends on specificity and accuracy. The evaluation of an instrument's precision guarantees a safe threshold for the beginning of biological or pathological bias and, thus, for the significance of nuclear DNA analyses. Determinations of precision with image microphotometry have been performed previously [22]. Thirty measurements of one prophase from a cancer cell line showed a CV of 0.01, and a complete telophase yielded CV = 0.02. Both minor scatterings meant high precision. Samples of more than 80 mitotic prophases repeatedly showed CVs = 0.05, indicating that the instrument's precision was diminished only by variabilities from individual cells and the optical environment.

Image microphotometry has encountered serious competition from flow fluorometry because the latter offers rapid measurements with high precision. However, a lack of accuracy may be inevitable when solid tissues are disintegrated [23].

Completing the S period of the mitotic cycle, the cell nucleus has obtained a set of four chromatids (4.0 c) and maintains this DNA content until the mitotic anaphase equationally separates the chromosomes [24]. True mitotic figures were found using the light microscope in fetal tissues and in various inflamed adult tissues [22]. Their 4 c DNA content was confirmed by image microphotometry in prophases, metaphases and both parts of telophases. The dynamics of chromosome segregation demand more space, and 15 μm sections were found to be appropriate [18].

Precancerous lesions showed divisions with quantitative aberrations from sound 4.0 c DNA content, reflecting the onset of genomic instability. The pathologically injured entities have previously been termed CDFs [18], some of which were found at 3.5 c and even below. However, the abundant mass of CDFs showed DNA contents raised beyond the mitotic 4.0 c level, and these were evaluated with the 4.5 c ER (Figure 8). This close threshold is justified by the high precision obtained from true mitoses.

The onset of CDFs was detected before the DNA aneuploidy of interphase nuclei had been established. CDFs first appear in low grade dysplasias; their frequency is increased in high grade dysplasias and malignant lesions [19, 22]. These observations were the key to the significance of CDFs, which are understood as a sure indication and measure of the genome's instability. Further confirmation came from the unbalanced telophase hemispheres, particularly in precancers and cancers of uterine cervix, skin, oral mucosa, stomach, colon adenoma and ulcerative colitis.

Breast cancer was chosen for testing the reliability of interphase and CDF measurements, because cases with well, moderately and poorly differentiated types occur frequently. The poorly differentiated type with DNA aneuploidy in interphases enriched the CDFs to 82.2%. The well differentiated type was confirmed with diploid profiles from interphase nuclei and showed 29.3% CDFs defined by the 4.5 c ER. This surprising result may be interpreted as an early sign of progression. While the histology and the interphase analysis exhibited a well-differentiated cancer with a diploid interphase population, the fraction of CDFs (Table 1, Figure 8) might trigger general DNA aneuploidy in the forthcoming development.

The reproducible records from CDFs in precancer and cancer have confirmed our finding with interphase nuclei that the choice of sufficient depth for microsections makes any data correcting algorithm unnecessary.

1. Caspersson T. Methods for the determination of the absorption spectra of cell structures. *J R Microscop Soc* 1940, **60**, 8–25.
2. Feulgen R, Rossenbeck H. Mikroskopisch-chemischer Nachweis einer Nucleinsäure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. *Z Physiol Chem* 1924, **135**, 203–248.
3. Bauer H. Die Feulgensche Nuklealfärbung in ihrer Anwendung auf cytologische Untersuchungen. *Z Zellf* 1932, **15**, 225–247.
4. Caspersson T. Die quantitative Bestimmung von Thymonucleinsäure mittels fuchsinschwefliger Säure. *Biochem Z* 1932, **253**, 97–110.
5. Vendrely R, Vendrely C. The results of cytophotometry in the study of the deoxyribonucleic acid (DNA) content of the nucleus. *Intern Rev Cytol* 1956, **5**, 171–197.
6. Würthner K, Sachs H, Bahnsen J. Zum Problem der Kernanschnitte bei der Zytophototmetrie an histologischen Präparaten. *Histochemie* 1972, **32**, 261–270.
7. Tavares AS, Costa J, Costa-Maia J. Correlation between ploidy and prognosis in prostatic carcinoma. *J Urol* 1973, **109**, 676–679.
8. Kreicbergs A, Zetterberg A. Cytophotometric DNA measurements of chondrosarcoma: methodologic aspects of measurements in tissue sections from old paraffin-embedded specimens. *Anal Quant Cytol* 1980, **2**, 84–92.
9. Berryman I, Sterrett GF, Papadimitriou JM. Feulgen DNA cytophotometry in histologic sections of mammary neoplasms. *Anal Quant Cytol* 1984, **6**, 19–23.
10. Bins M, Takens F. A method to estimate the DNA content of whole nuclei from measurements made on thin tissue sections. *Cytometry* 1985, **6**, 234–237.
11. Bauer HCF, Kreicbergs A, Tribukait B. DNA microspectrophotometry of bone sarcomas in tissue sections as compared to imprint and flow DNA analysis. *Cytometry* 1986, **7**, 544–550.
12. Askensten UG, Moberger B, Auer GU. Methodological aspects on cytochemical DNA assessments of adenocarcinoma in the endometrium by means of image and flow cytometry using conventional formalin-fixed and paraffin-embedded specimens. *Arch Geschwulstforsch* 1990, **60**, 209–216.
13. Epstein JI, Christensen WN, Steinberg GD, Carter HB. Comparison of DNA and nuclear size, shape and chromatin irregularity in tissue sections and smears of prostatic carcinoma. *Anal Quant Cytol Histol* 1990, **12**, 352–358.
14. Klapperstück T, Wohlrab W. DNA image cytometry on sections as compared with image cytometry on smears and flow cytometry in melanoma. *Cytometry* 1996, **25**, 82–89.
15. McCready RW, Papadimitriou JM. An analysis of DNA cytophotometry on tissue sections in a rat liver model. *Anal Quant Cytol* 1983, **5**, 117–123.
16. Bacus JW, Bacus JV. A method of correcting DNA ploidy measurements in tissue sections. *Mod Pathol* 1994, **7**, 652–664.
17. Hansemann D. Ueber pathologische Mitosen. *Arch path Anat Phys klin Med* 1891, **123**, 356–370.
18. Steinbeck RG. Atypical mitoses in lesions of the oral mucosa: A new interpretation of their impact upon tumorigenesis. *Oral Oncol* 1997, **33**, 110–118.
19. Steinbeck RG. The DNA content of chromosome division figures and interphase nuclei classifies ulcerative colitis. *Eur J Cancer* 1998, **34**, 175–181.
20. Auer G, Caspersson T, Wallgren A. DNA content and survival in mammary carcinoma. *Anal Quant Cytol* 1980, **3**, 161–165.

21. Zetterberg A, Esposti PL. Prognostic significance of nuclear DNA levels in prostatic carcinoma. *Scand J Urol Nephrol* 1980, **55**, 53–58.
22. Steinbeck RG. Imbalance of morphologically addressed telophases reflects interphase DNA aneuploidy in tumorigenesis. *Eur J Histochem* 1997, **41**, 243–254.
23. Steinbeck RG, Heselmeyer KM, Neugebauer WF, Falkmer UG, Auer GU. DNA ploidy in human colorectal adenocarcinomas. *Anal Quant Cytol Histol* 1993, **15**, 187–194.
24. Roux W. *Ueber die Bedeutung der Kerntheilungsfiguren. Eine hypothetische Erörterung*. Leipzig, Wilhelm Engelmann, 1883.

Acknowledgements—Ms Mia Thorsell, Stockholm, and Mr Uwe Koester, Flensburg, have given excellent technical assistance. Ms Jayne Welling-Wolf, Kiel (Germany), provided linguistic aids. Supported by the Swedish Cancer Society, Stockholm, and the Research Funds of the Institute of Pathology, Flensburg (Germany).