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Flow cytometric bivariate analysis of DNA and cytokeratin in colorectal cancer

Carolin Post [a], Ib Jarle Christensen [a], Henrik Flyger [b], Jette Christiansen [a] and Jørgen K. Larsen [a,*]
[a] *Finsen Laboratory, Finsen Center, Rigshospitalet, University Hospital, Copenhagen, Denmark*
[b] *Department of Surgery, Hillerød Hospital, Hillerød, Denmark*

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Abstract. Different opinions about flow cytometric estimates of DNA aneuploidy and/or S-phase fraction (SPF) as supplementary prognostic markers in colorectal cancer are to some degree associated with methodology. Using univariate DNA analysis, we have previously investigated the DNA ploidy in colorectal cancer, its heterogeneity within and between tumors and its relation to survival. To improve detection of DNA aneuploid subpopulations and particularly estimation of their SPF's we investigated a method for bivariate DNA/cytokeratin analysis on fine-needle aspirates of 728 frozen biopsies from 157 colorectal tumors. Unfixed aspirates were stained with propidium iodide and FITC-conjugated anti-cytokeratin antibody in a saponin-buffer. A significant association between SPF and debris was observed. There were no substantial difference in DNA ploidy patterns between univariate and bivariate measurements (concordance was 92–95%). No new DNA aneuploid subpopulations were detected in cytokeratin-gated compared to ungated or univariate histograms. Debris-adjusted SPF's of cytokeratin-gated histograms were significantly higher than of ungated histograms, also for subpopulations with DI > 1.4 ($p < 0.0001$). There was no significant association between SPF and survival.
Keywords: Colorectal cancer, DNA aneuploidy, S-phase fraction, cytokeratin, flow cytometry, prognosis

1. Introduction

Colorectal cancer is a common cancer and about 50% of patients who undergo curative surgery die of recurrent disease within a 5 year period. The best prognostic marker is Dukes' staging. However, in Dukes' stage B and in Dukes' stage C the survival of patients and recurrence of disease are highly variable. Therefore, other parameters indicating whether these patients would benefit from adjuvant treatment are needed. There has been some dispute as to whether flow cytometric estimates of DNA aneuploidy and/or cell cycle fractions (S or $S + G_2 + M$) are useful as supplementary prognostic markers. The different conclusions are to some degree associated with the methodologies applied [2]. Many recent investigations indicate a prognostic significance of flow cytometric DNA ploidy analysis [5,6,21,23,25,37] and SPF analysis [6,21,23,27], while some others do not [26,38]. The contradictions may be caused by differences between individual investigations regarding the number of patients, clinical stage, tissue sampling strategy, use of fresh or paraffin embedded material, staining procedure, analytical hardware and software. When solely based on univariate DNA flow cytometry, the success in detection and quantification of aneuploid subpopulations and cell cycle fractions is limited by the DNA measurement precision and statistical methods for resolving the DNA histogram, and depends on calibration with internal DNA reference cells and correction for background debris and aggregates [2,27].

A complicating factor in DNA flow cytometry of colorectal tumors is the limited possibility for resolving the tumor heterogeneity. DNA clonal heterogeneity, defined as two or more DNA aneuploid stemlines in the same tumor, is well established, however, most studies have been based on only one biopsy from each tumor. Many investigators have pointed out the importance of taking multiple samples from each tumor to detect all coexisting subpopulations [8,19,33].

*Corresponding author: Jørgen K. Larsen, The Finsen Laboratory, Rigshospitalet, Dept. 8621, Strandboulevarden 49, DK-2100 Copenhagen, Denmark. Tel.: +45 35455750; Fax: +45 35385450; E-mail: j.k.larsen@finsenlab.dk.

In order to increase the prognostic significance of flow cytometric DNA analysis it has been recommended to include parameters associated with cell proliferation and differentiation [1,2]. Estimates of SPF may not be conclusive for cell proliferation, because also quiescent or dying cells may reside with S-phase DNA content. The application of *in vivo* labelling with halogenated deoxyuridine for detection of DNA replicating cells and calculation of cell kinetic parameters [11,18] is limited by ethical reasons and rather difficult to arrange in multicenter studies. Discrimination of the cancer cells may be increased by combining staining of DNA with immunochemical staining of cytokeratin. This enables the exclusion of cytokeratin-negative, non-epithelial cells and thus improves detection of individual DNA aneuploid subpopulations and estimation of their SPF's. Cell suspensions for combined DNA and cytokeratin analysis have usually been prepared from fresh tissue and fixed before staining [4, 38], but have also been prepared from paraffin embedded tissue [13,14].

Using flow cytometric univariate DNA analysis according to the method of Vindeløv et al. [28], we have recently investigated the DNA ploidy in colorectal adenocarcinomas of 163 patients, each tumor represented with up to 5 biopsies [8]. In the present study, we compare results from flow cytometric bivariate DNA/cytokeratin analysis and univariate DNA analysis. Both analyses were performed on a material of additional frozen aliquots of fine needle aspirates from the tumors investigated previously. For DNA/cytokeratin analysis, we adopted the procedure of Jacob et al. [10] for immunochemical staining of unfixed cells after permeabilization with saponin. By avoiding fixation we hoped to retain the same high DNA measurement precision and stoichiometry and minimal cellular aggregation as obtained with the method of Vindeløv et al. [28].

Our primary aim was to investigate whether cytokeratin gating based on this procedure for DNA/cytokeratin staining would improve the detection of DNA aneuploid subpopulations and in particular the estimation of their SPF's. Secondly, we wanted to investigate the possible prognostic significance of these parameters.

2. Materials and methods

2.1. Tumor biopsies

Our material consisted of fine-needle aspirates of tumor biopsies from 157 patients with colorectal cancer. The patients were consecutively included as a part of a prospective, double-blind study, randomised to either placebo or ranitidine treatment (RANX05 multicenter study). There was no statistically significant difference in survival between the two treatment groups [17]. There were 83 males and 74 females included, with a median age of 68 years (range 35 to 87 years). The patients were staged by Dukes' classification. The patients had been followed for median 79 months (range 68 to 86). All tumors were histologically classified as adenocarcinomas. From each fresh surgical specimen, biopsies were taken from each of the four corners and from the center of the tumor, if possible. Biopsies were frozen at -80°C.

For validation of the quality of the above material of long-stored colorectal tumor biopsies and aspirates, measurements were done on fresh, short-stored colorectal tumor tissue from additional 20 patients and on non-cancerous colorectal mucosa biopsies from 14 patients.

2.2. Cell preparation

Fine-needle aspirates were taken from thawed biopsies with a gauge 23 needle, aiming at a total of approximately 5×10^6 aspirated cells per biopsy. The aspirate was suspended in cold citrate buffer (250 mM sucrose, 5% DMSO, 40 mM sodium citrate, pH 7.6), divided into 5 cryotubes, frozen and stored at -80°C [29]. At the day of measurement the aspirate was thawed, resuspended, and filtered through a 102 μm monofilament polyester mesh (PE-102-HC, SEFAR, Rüschlikon, Switzerland). To increase the recovery of filtered cells, 300 μl of cold citrate buffer was sequentially added to the filter. The following preparations were all done on ice-bath. The filtered cell suspension was divided into two portions: one fifth volume to be stained for univariate DNA measurements, and four fifth volume to be stained for bivariate DNA/cytokeratin measurements. Both portions were centrifuged (1,250 g, 5 min, 4°C), and the supernatant carefully aspirated, leaving a residual volume of 50–100 μl with the cell pellet.

For univariate DNA analysis, the cells were stained according to the method of Vindeløv et al. [30], by sequential addition of 250 μl of Vindeløv et al.'s solution A (10 min at room temperature), 187 μl of solution B (10 min at room temperature), and 187 μl of solution C (at least 15 min on ice).

For bivariate DNA/cytokeratin analysis, the cells were stained according to a modification of the method of Jacob et al. [10]. First, the cell pellet was resuspended by addition of 500 μl (to cell-rich samples) or 400 μl (to cell-poor samples) of a DNA-staining solution: 0.3% saponin (S-2149, Sigma, St. Louis, Missouri, USA), 50 μl/ml propidium iodide (PI; P-4170, Sigma), and 0.2 mg/ml RNase (R-4875, Sigma), in PBS (Dulbecco's phosphate buffered saline without Ca and Mg, pH 7.2). From the cell-rich samples a portion of 100 μl was reserved for staining with a negative control antibody. Then, after 10 min, the samples were immunochemically stained by addition (without previous washing) of 10 μl of FITC-conjugated anti-cytokeratin antibody (clone MNF116, IgG_1, F-0859, DAKO, Glostrup, Denmark), or 5 μl FITC-conjugated negative control antibody (IgG_1, X-0927, DAKO), respectively.

Chicken (CRBC) and trout (TRBC) red blood cells were used as internal references [31], but were first added after an estimate of the cell count had been obtained in the flow cytometer. The CRBC and TRBC were stained separately, but according to the same protocols as for the tumor cells. The final measurement was done at least 30 min after addition of the references. The overall staining time before measurement was 30–180 min.

2.3. Flow cytometry

For flow cytometric measurements a FACSort (Becton Dickinson, San José, California, USA) was used. List mode acquisition included forward and orthogonal light scatter, FITC fluorescence (FL1-log; 530/30 nm), PI fluorescence (FL2-height and -area; 585/42 nm), and time. PI fluorescence was used as threshold trigger at the level of approximately 10% of the DNA diploid FL2-height level. Approximately 10,000 counts were acquired for univariate DNA distributions (FL2-area). For bivariate DNA/cytokeratin distributions 50,000 counts were acquired in samples stained with anti-cytokeratin antibody (so that cytokeratin-positive and -negative subsets each included sufficient cells for SPF estimation) and 10,000 in those stained with negative control antibody. In accordance with the number of counts to be collected, the sample flow rate was "low" for univariate DNA measurements and "medium" for bivariate DNA/cytokeratin measurements. During bivariate DNA/cytokeratin measurements the compensation for spectral overlap between FITC and PI fluorescence (FL2–FL1) was set to 6%. For bivariate DNA/cytokeratin analysis the list mode files were gated according to (1) elapsed time for control of instrumental drift, (2) PI fluorescence pulse shape (FL2-height/FL2-area) for elimination of doublets, and (3) FITC fluorescence (FL1-log) for selection of cytokeratin-expressing cells, by comparison with samples stained with isotype-matched negative control antibody.

2.4. Statistics

The observed histograms (ungated, gated and univariate) were deconvoluted by maximum likelihood as described by Vindeløv and Christensen [28]. The analysis of the ungated, gated and univariate histograms were processed blindly, that is without knowledge of the results on the other histograms from the same biopsy. The covariates of the fitted model were: the CV of the G_1 peaks, the proportion in each subpopulation and their G_1, S and $G_2 + M$ cell cycle fractions as well as the G_1 peak mean. Debris was fitted by a truncated exponential function extrapolated to include all subpopulations. The DI's were calculated from the G_1 peak means using the CRBC and TRBC references in the sample. The S-phase cells were assumed to be uniformly distributed. A fitted model was considered valid if the fit was good. A DNA index was defined to be DNA diploid if it was within the 95% confidence interval as defined by the lymphocyte standard (1.00 ± 0.05). Only DNA subpopulations with a G_1 peak representing at least 10% of the total count in the region of interest (i.e., not including debris or internal reference cells) were considered. From DNA distributions with more than one DNA subpopulation, total SPF's were calculated as the weighted mean of the SPF's. A similar calculation was done for DNA aneuploid subpopulations with $DI > 1.4$ with the constraint that the fraction of subpopulations with $DI > 1.4$ must be at least 25% of the sample. The DNA ploidy patterns of ungated and gated histograms as well as univariate histograms derived from the same biopsy were considered to be concordant if DNA subpopulations with the same DNA index (with a small margin for statistical error) were detected in each histogram type. Statistical calculations (except deconvolution of histograms) and data management were done using the SAS® software (version 8.01; SAS Institute, Cary, NC, USA). P-values less than 0.05 were considered significant.

2.5. Selection criteria

A DNA histogram (ungated, gated or univariate) was included for further analysis if the following criteria were fulfilled:

The CV of the G_1 peaks was $<5\%$.

The number of counts in a gated histogram as well as univariate histograms was at least 2,000 in the region of interest.

The number of counts classified as debris was $<50\%$ of the number of counts in the region of interest.

3. Results

3.1. Selected data

Bivariate DNA/cytokeratin measurements were done on 728 biopsies (representing 157 patients) and univariate DNA measurements were done on 649 biopsies (152 patients). Applying the above described technical criteria yielded valid univariate measurements on 288 biopsies representing 105 patients, and from bivariate measurements on 389 biopsies representing 127 patients. For the selected dataset, the median CV of DNA histograms was 2.2% (range 1.1–4.9%) for univariate measurements and 3.0% (range 1.6–5.0%) for bivariate cytokeratin measurements. The median debris fraction was 15.9% (range 0.9–49.9%) for univariate measurement and 26.9% (range 2.5–50.0%) for bivariate cytokeratin measurement. The median number of counts in the region of interest was 9,133 (range 2,073–34,734) in cytokeratin-gated DNA histograms and 6,140 (range 2,006–30,831) in univariate DNA histograms.

For the 127 patients that were represented in the selected dataset from bivariate measurements, the distribution by Dukes' stage (12 patients in stage A, 45 in stage B, 48 in stage C, and 22 in stage D), gender, tumor location, age and deaths was similar to what was found in our previous investigation [8]. However, the number of biopsies per patient was lower than previously: now 26 patients were represented by 5 biopsies, 33 by 4 biopsies, 19 by 3 biopsies, 21 by 2 biopsies, and 28 by 1 biopsy. There was no evidence that shortage of material was associated with Dukes' stage or with the selection criteria.

3.2. Validation of the quality of biopsy material

The fresh, shortly stored colorectal tumor tissue had a median CV of 2.8% (range 1.7–6.2%) in DNA histograms from DNA/cytokeratin measurements and 2.4% (range 1.5–3.8%) from univariate measurements.

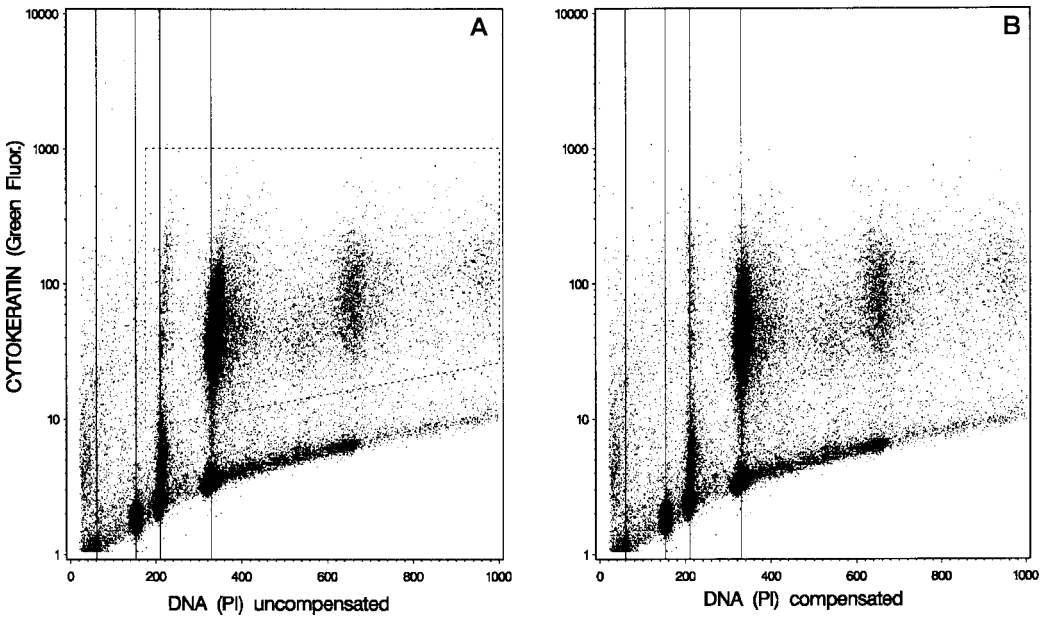

Fig. 1. Dot plot showing gating on cytokeratin stained cells (cytokeratin-positive within the dashed frame) before (A) and after compensation for spectral overlap (B). The compensation was based on regression analysis for the subset of G_1 cells with green fluorescence as the independent variable and red fluorescence as the dependent variable. The vertical lines indicate from left to right the G_1 peak positions of chicken and trout red blood cells, DNA diploid and DNA aneuploid cells.

Median debris fractions were 18% (range 2.9–46%) for DNA/cytokeratin measurements and 13% (range 3.8–29%) for univariate DNA measurements.

Analysis of non-cancerous colon tissue showed only diploid DNA indices, and no DNA aneuploid subpopulations were found.

Differences in total SPF between cytokeratin-gated and ungated measurements were not significant (paired t-test).

3.3. DNA ploidy distribution

The spectral fluorescence compensation during measurement was not sufficiently accurate and therefore the FL2-area coefficient of variation (CV) of the major G_1 peak was retrospectively minimized, in order to obtain the final DNA histograms for further analysis (Fig. 1). An example of the resulting DNA histograms (FL2-area, 50,000 counts), ungated as well as gated (cytokeratin-positive) in FITC fluorescence, is shown in Fig. 2.

Figure 2 also shows an example of the DNA ploidy heterogeneity between different biopsies from the same patient. For subpopulations with G_1-phase nuclei comprising >10% of counted nuclei, we found total concordance between the occurrence of DNA aneuploid subpopulations in univariate and bivariate measurements in 92% of all biopsies, and partial concordance in all biopsies. Comparing cytokeratin-gated and ungated measurements, the concordance was 95%. No new DNA aneuploid subpopulations were detected from cytokeratin-gated measurements, as compared to ungated and univariate measurements.

The median size of the DNA aneuploid fractions was 53% for ungated histograms (only those with DNA aneuploid components), 66% for gated histograms and 71% for univariate histograms. The gated histograms showed significantly higher aneuploid fractions ($p < 0.0001$, paired t-test) compared to ungated histograms.

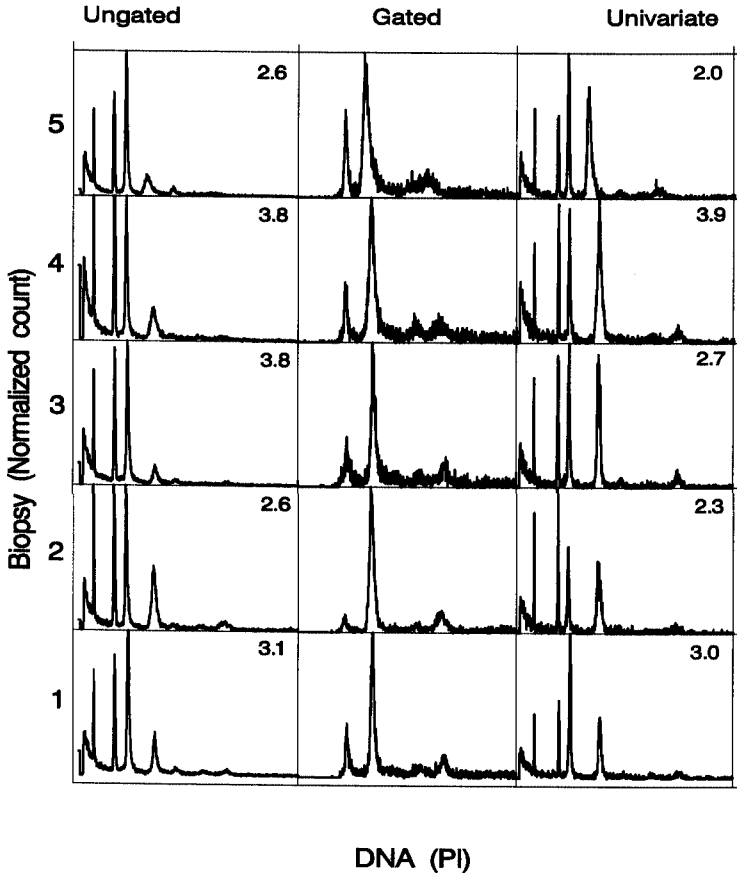

Fig. 2. Cytokeratin-ungated, cytokeratin-gated (positive) and univariate DNA histograms of one patient, represented with 5 tumor biopsies. The aneuploid peak is clearly higher in cytokeratin-gated than in ungated, in histograms of all of the 5 biopsies. The CV is indicated in the upper right corner.

The univariate histograms showed non-significantly higher aneuploid fractions than gated histograms ($p = 0.05$, paired t-test).

3.4. SPF distribution

The median total SPF for ungated histograms from all biopsies was 22% (range: 1–47%), 25% (range: 5–63%) for gated histograms and 19% (3–40%) for univariate histograms. Paired analysis showed that SPF estimates from gated histograms were significantly higher than ungated (mean difference 3%, $p < 0.0001$, paired t-test). And ungated histograms were significantly higher than univariate (mean difference 3%, $p < 0.0001$, paired t-test).

The median aneuploid SPF (SPF of subpopulations with DI > 1.4 and size $\geqslant$25%) was 31% for ungated histograms, 34% for gated histograms and 22% for univariate histograms. There was no statistical difference demonstrated between the aneuploid SPF estimates from ungated and gated histograms ($p = 0.78$). There was however a significant difference between the aneuploid SPF from bivariate and univariate histograms ($p < 0.0001$, paired t-test).

A significant association between the estimated SPF and the debris fraction was observed. Figure 3 shows the association between total SPF and the debris fraction. This suggested an adjustment of the SPF for debris using linear regression. The results of this adjustment are shown in Table 1. The mean differences between ungated, gated and univariate histograms are shown in Table 2, indicating that gated histograms have significantly higher aneuploid SPF's than ungated in contrast to the unadjusted SPF's.

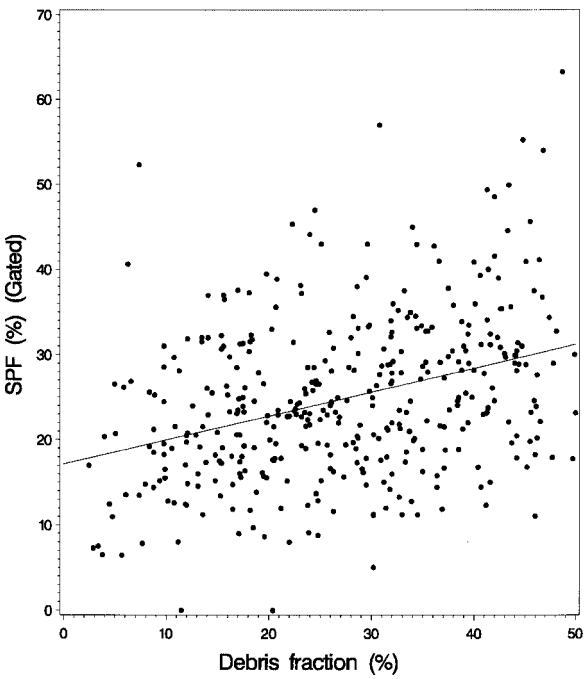

Fig. 3. Total SPF in cytokeratin-gated DNA histograms: Plot shows SPF as a function of debris counts, regression is $17.2 + 0.28\times$ debris.

The total and aneuploid SPF for each patient was also calculated by the mean. The results on the SPF by patient are shown in Table 1, including the adjusted SPF values.

3.5. Survival analysis

The mean values of the total SPF's and the SPF's of subpopulations with DI > 1.4 in the gated histograms were calculated for each patient. The total SPF's were grouped using the tertiles to define cut-points and the overall survival was calculated for each group by the Kaplan–Meier method. The results are shown in Fig. 4A. The same analysis applied to the adjusted SPF values is shown in Fig. 4B. The SPF's of subpopulations with DI > 1.4 were dichotomized, and the results are shown in Fig. 4C and 4D, unadjusted and adjusted, respectively. There is no significant effect of SPF in this study. However, there is a trend towards better prognosis for patients with high unadjusted SPF of subpopulations with DI > 1.4 (Fig. 4C).

4. Discussion

4.1. Validation of the method for DNA/cytokeratin analysis

In previous investigations with flow cytometric DNA/cytokeratin analysis in colorectal cancer the biopsy material was disaggregated mechanically or enzymatically into suspensions of intact cells, these were fixed, and the content of cytokeratins 8, 18 and 19 was stained using the CAM 5.2 antibody (Becton Dickinson), a method that requires processing within 24 hours after surgical resection to avoid deterioration of the histograms [38,39]. Alternatively, DNA/cytokeratin analysis was performed on paraffin embedded material, resulting in a low DNA CV and relatively high estimates of SPF [13,14]. In multicenter studies it is practical to freeze the biopsies for later processing. A recent study has demon-

Table 1
Estimates of S-phase fraction (SPF, %) adjusted to minimize the influence of debris

Type of DNA histogram	Mean ± SD	Range	Median	*N*
A. Biopsies				
Total SPF (% of cells)[a]				
Univariate	14 ± 6	0–32 (3–40)[b]	14 (19)[b]	288
Cytokeratin-ungated	15 ± 7	0–39 (1–47)	15 (22)	389
Cytokeratin-gated	17 ± 9	0–50 (5–63)	17 (25)	389
SPF of subpopulations with DI > 1.4 (% of cells)[c]				
Univariate	14 ± 9	0–38	15 (22)	105
Cytokeratin-ungated	18 ± 13	0–60	17 (31)	122
Cytokeratin-gated	25 ± 15	0–61	24 (34)	146
B. Patients				
Total SPF (% of cells)[a]				
Univariate	14 ± 5	0–29	14 (19)	105
Cytokeratin-ungated	15 ± 6	0–30	14 (21)	127
Cytokeratin-gated	16 ± 8	0–41	17 (24)	127
SPF of subpopulations with DI > 1.4 (% of cells)[c]				
Univariate	15 ± 10	0–38	15 (24)	42
Cytokeratin-ungated	19 ± 12	0–47	17 (33)	57
Cytokeratin-gated	26 ± 15	0–61	25 (34)	65

[a]The weighted sum of all S-phase fractions in one biopsy.
[b]In parenthesis, estimate of SPF not adjusted with regard to the SPF/debris function shown in Fig. 3.
[c]Defined as the weighted sum of the SPFs of those subpopulations in one biopsy that have a DI $\geqslant$ 1.4 and each comprise at least 25% of the number of counts in the region of interest.

Table 2
Estimates of the mean difference of the adjusted SPF between univariate and bivariate DNA histograms

A. Total SPF[a]	
Cytokeratin-ungated minus univariate	2% (SD = 6%; $p < 0.0001$)[b]
Cytokeratin-gated minus ungated	2% (SD = 6%; $p < 0.0001$)
B. SPF of subpopulations with DI > 1.4[c]	
Cytokeratin-ungated minus univariate	4% (SD = 11%; $p = 0.007$)
Cytokeratin-gated minus ungated	6% (SD = 11%; $p < 0.001$)

[a]The weighted sum of all S-phase fractions in one biopsy.
[b]Paired *t*-test.
[c]For subpopulations with DI > 1.40 and G_1 fraction of >25% of number of counts in the region of interest.

strated the validity of automated tissue disaggregation with the Medimachine (DAKO) on freeze-stored biopsy material, followed by fixation of the cell suspension with methanol [4]. In the present study, we have performed the DNA/cytokeratin analysis on a material of fine-needle aspirates from tumor biopsies collected from several hospitals [8]. Tumor biopsies as well as aspirates were freeze-stored. Cells were permeabilized with saponin [10] and cytokeratin was stained using the pan-cytokeratin antibody MNF116 (DAKO), specific for cytokeratins 5, 6, 8, 17 and 19.

In a pilot experiment we mixed cells from a DNA aneuploid colorectal cancer cell line (COLO-201, COLO-205 and LoVo) with cells from a DNA near-diploid leukemic cell line (HL-60) before DNA/cytokeratin staining. Permeabilization with saponin and staining with FITC-conjugated MNF116 antibody resulted in a similar discrimination of cytokeratin-positive cells in terms of the fluorescence intensity and fraction of positive cells, as we found for permeabilization with alcohols and/or staining with FITC-conjugated CAM 5.2 antibody. In addition, DNA measurement precision was higher and cellular aggregation

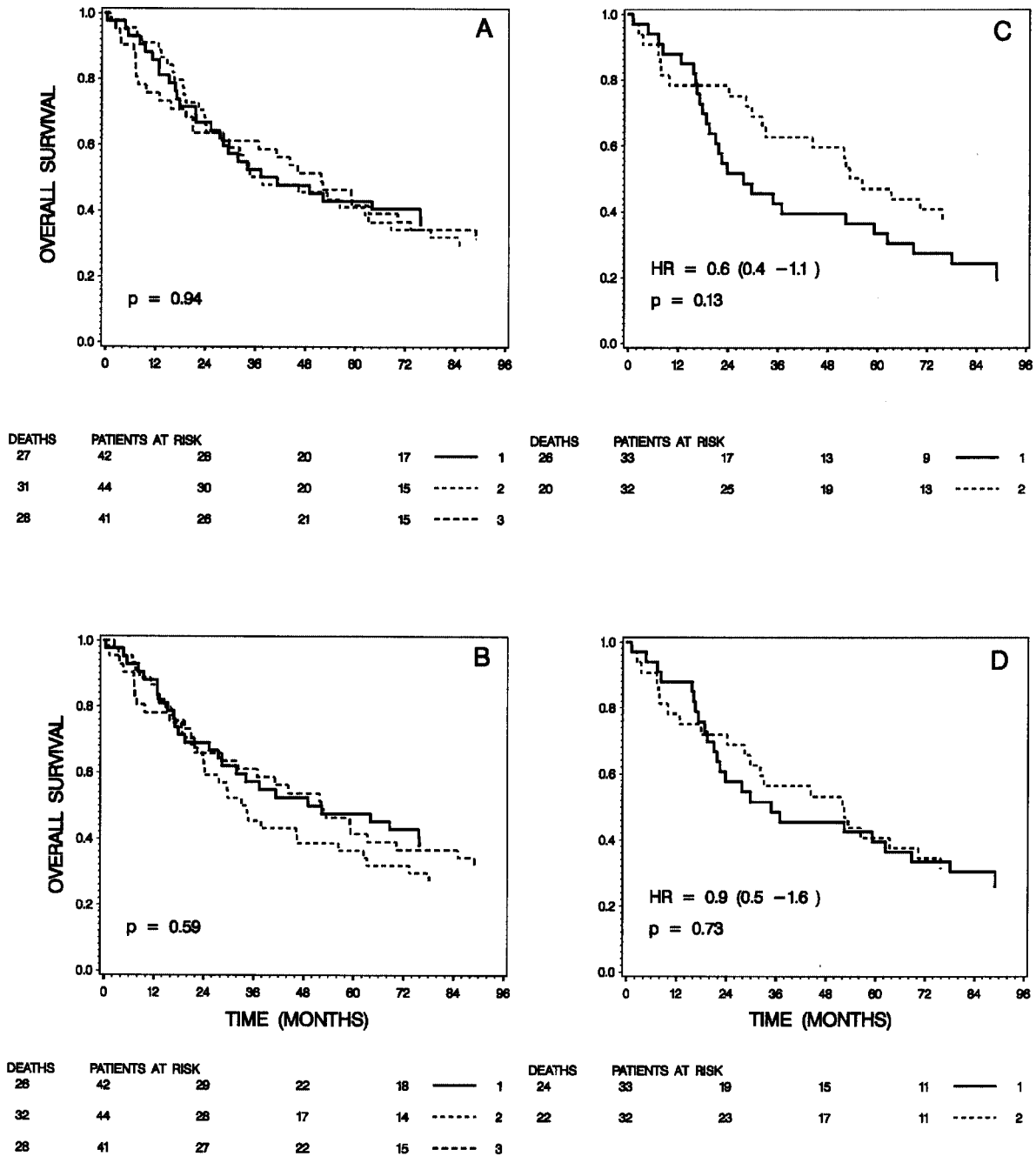

Fig. 4. Kaplan–Meier survival plots for 127 patients, under each curve the number of events (deaths) are shown to the left and the number of patients at risk at 0, 24, 48 and 72 months are shown below the curve for each stratum. (A) Total unadjusted SPF categorized by tertiles (1: <21%, 2: 21–27%, 3: >27%). (B) Total adjusted SPF categorized by tertiles (1: <12%, 2: 12–20%, 3: >20%). (C) Unadjusted SPF of subpopulations with DI > 1.4 dichotomized by the median SPF (1: ⩽34%, 2: >34%). (D) Adjusted SPF of subpopulations with DI > 1.4 dichotomized by the median SPF (1: ⩽25%, 2: >25%). For (C) and (D), the hazard ratio (HR) with its 95% confidence interval is shown.

less in analysis of unfixed cells (data not shown). However, our pilot experiment on cell lines may not correspond to the practical situation for disaggregation and staining of solid tissues, where the retention of cytokeratin may be critical [35]. Altogether, the treatment with freezing, thawing, fine-needle aspiration and saponin may adversely result in disruption of many cells, partly or entirely loosing their cytokeratin. This is indicated in Fig. 1 by the numerous DNA aneuploid cells or nuclei not showing any cytokeratin content. In the present study we assumed that this damage was not selective, but randomly distributed over the subpopulations. As expected, the median size of the DNA aneuploid fraction was higher for cytokeratin-gated (66%) than ungated measurements (53%). However, the DNA aneuploid fraction reached its highest level (71%) in univariate DNA measurements. This may at least partly be explained by the different DNA measurement precision between the univariate and bivariate DNA analyses.

The need for fluorescence compensation would have been largely reduced, if FL3-area (650 nm long pass) had been used instead of FL2-area (585/42 nm band pass) for collection of propidium iodide fluorescence. However, because DNA measurement precision and accuracy are of utmost importance for the optimal detection and quantification of DNA aneuploid subclones, FL2-area was chosen for the measurement of propidium iodide fluorescence, since it could be adjusted to higher precision than FL3-area. For optimization of the accuracy, it then became important, besides the scale calibration for non-linearity, to apply an objective method for reduction of the FITC fluorescence contribution to the FL2-area signal, based on retrospective compensation (Fig. 1).

Debris compensation is necessary for adequate quantification of DNA aneuploid subpopulations and their SPF's [34]. Therefore, the DNA histograms were first corrected by subtraction of the distribution of counts of debris, fitted by an exponential curve between the left part of the histogram and extrapolated to include all DNA subpopulations. As a precaution in estimation of the SPF values, we found it necessary to introduce a further adjustment based on the SPF-debris regression function (Fig. 3). It is interesting that this association between SPF and debris was found in univariate as well as bivariate measurements. It remains an unsolved question to what extent this debris is technical and incompletely corrected for in the histogram deconvolution, or biological and associated to high levels of apoptotic cell degradation. A technical reason is indicated by the fact that there was more debris in the long-stored than in the shortly stored samples. Adjustment by the SPF-debris regression function overrules the possibility of using SPF for indicating an increased level of cell proliferation combined with cell degradation. However, this study was not designed for specific measurements of cell proliferation and cell degradation. We found no occurrence of apoptotic sub-G_1 peaks in neither the univariate nor bivariate measurements.

4.2. Tumor heterogeneity, DNA aneuploidy, SPF, and survival

In the study of colorectal tumors it is important to take into account the explicit tissue heterogeneity that appears as a mosaic pattern of multiple DNA aneuploid subpopulations [19,24]. Therefore, studies of the prognostic significance of DNA aneuploidy must include multiple samples from each tumor. In our previous flow cytometric study by univariate DNA analysis, where each tumor was represented with up to 5 biopsies, DNA aneuploidy was detected in tumors from 89% of patients, whereas only 11% of patients had a solitary DNA diploid cell population (DI 1.00 ± 0.03) [8]. In contrast, many investigators have reported a much higher fraction of DNA diploid colorectal tumors, in the range of 34–77% [5,9,21,23,25,26,36–38]. This may be associated with differences in patient material, number of biopsies per patient, DNA measurement precision, or methods for histogram deconvolution.

In our previous study based on univariate DNA analysis, the distinction between DNA diploidy and aneuploidy did not predict survival [8]. However, grouping subpopulations into three DI categories (group 1: DI 0.97–1.15, group 2: DI 1.15–2.06, and group 3: DI < 0.97 and/or DI > 2.06) showed a significant difference in survival in a Cox multivariate analysis including Dukes' stage ($p = 0.049$ comparing group 2 to 1, and $p = 0.01$ comparing group 3 to 1). Other investigations have confirmed that the distinction between different DI classes is more relevant for prognostic indication than the mere distinction between DNA diploid and aneuploid [25,37]. The DI distribution and the extent of intra-tumor heterogeneity we found in the present study are in high concordance with the results from our previous study on the same set of biopsies. An important result is that no new DNA aneuploid subpopulations were found in this study, neither by the univariate nor bivariate measurements.

Several investigations based on univariate DNA analysis found that the SPF (or $S + G_2 + M$ fraction) is higher for DNA aneuploid than for diploid colorectal tumors [3,4,7,16,20]. In a study of 157 patients Kouri et al. found considerable heterogeneity of SPF in multiple samples from the same tumor, with an average of 29% difference between the highest and lowest SPF [12]. Both Enker et al. [7] and Mazzei et al. [16] found that higher SPF was not significantly correlated to worse prognosis on survival. However, Witzig et al. [36] found prognostic significance of DNA aneuploidy as well as $S + G_2 + M$ fraction based on DNA histograms from paraffin-embedded tumor tissue from 694 patients with colorectal stage B2 or C adenocarcinomas, but because of high CV the distinction between diploid and aneuploid G_1 peaks and the cell $S+G_2+M$ regions may be questionable.

Because the benefit of flow cytometric cytokeratin/DNA analysis in discriminating DNA aneuploid tumor cells subpopulations and estimating their DI and cell cycle distribution has been established for prognostic purposes in other solid cancers, it could be assumed that this technique might also be of advantage in studies of colorectal cancer. Thus, Zarbo et al. [38] conducted an investigation on a prospective series of 309 human colorectal carcinomas with 4–6 years of follow-up. Fresh tumors were mechanically dissociated into whole cell suspensions and stained for DNA/cytokeratin. Estimates of the $S + G_2M$ fraction were categorized by tertiles. However, DNA ploidy and $S + G_2M$ fraction measurements were not predictive of survival for the overall group or within any particular stage of colorectal carcinoma. However, differing from our material, adjuvant chemo- or radiotherapy was applied to patients in Zarbo et al.'s investigation.

In the present study, we tried to obtain high quality SPF estimates by using a method that provides a high DNA measurement precision together with staining of cytokeratin. Our estimates of SPF were significantly higher in cytokeratin-gated measurements than in ungated or univariate DNA measurements. This might imply that additional prognostic information could be obtained by DNA/cytokeratin measurements. After adjustment based on the SPF-debris regression function, our SPF estimates were in the same size range as those reported by many other investigators, e.g., Brockhoff et al. [4]. There was no significant association between the adjusted SPF and survival in this study. However, there was a difference in the unadjusted SPF of subpopulations with $DI > 1.4$, with high SPF having a better prognosis. The same tendency of better survival for patients with high SPF of subpopulations with $DI > 1.4$ was also found by Zarbo et al. [38]. A possible biological explanation for this might be that not all cells with S-phase DNA content necessarily are DNA-synthesizing, but some of them could be quiescent or dying. In experimental studies of solid tumors under hypoxic conditions, other investigators have demonstrated an accumulation of non-cycling cells with S- and G_2-phase DNA content [32]. In colorectal tumors, it has been shown that the flow cytometric $S + G_2 + M$ fraction does not correlate with the immunohistologic expression of the proliferation markers Ki-67 and PCNA [15]. And in breast cancer, an association has been demonstrated between the SPF and the fraction of cells showing apoptotic characteristics according to the TUNEL assay [22]. Regarding the present study, we cannot know to what proportions the debris in the DNA histograms was generated from: (1) fragments of cells that already underwent apoptosis and necrosis *in situ*, (2) quiescent or dying cells with S-phase DNA content and fragile to the staining procedure, or (3) damage inferred to the specimens during its collection and storage. For further investigation along these lines, more specific markers for the possible biological mechanisms should be applied, such as markers for detection of DNA-synthesizing cells by incorporation of halogenated deoxyuridines [11,18] and markers of apoptotic and necrotic cell degradation.

5. Conclusion

The ploidy pattern was very similar between the bivariate DNA/cytokeratin and the univariate DNA measurements. Thus there is no indication that the addition of measurements based on our DNA/cytokeratin protocol will provide new information on the DNA ploidy pattern.

The total SPF's as well as the SPF's of subpopulations with $DI > 1.4$ were significantly higher for cytokeratin-gated DNA measurements than for ungated or univariate DNA measurements.

There was no significant association between SPF and survival in this study.

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References

[1] Clinical practice guidelines for the use of tumor markers in breast and colorectal cancer. Adopted on May 17, 1996 by the American Society of Clinical Oncology, *J. Clin. Oncol.* **14** (1996), 2843–2877.

[2] K.D. Bauer, C.B. Bagwell, W. Giaretti, M. Melamed, R.J. Zarbo, T. E. Witzig and P.S. Rabinovitch, Consensus review of the clinical utility of DNA flow cytometry in colorectal cancer, *Cytometry* **14** (1993), 486–491.

[3] S. Bosari, A.K. Lee, B.D. Wiley, G.J. Heatley and M.L. Silverman, Flow cytometric and image analyses of colorectal adenocarcinomas. A comparative study with clinical correlations, *Am. J. Clin. Pathol.* **99** (1993), 187–194.

[4] G. Brockhoff, S. Fleischmann, A. Meier, F.P. Wachs, F. Hofstaedter and R. Knuechel, Use of a mechanical dissociation device to improve standardization of flow cytometric cytokeratin DNA measurements of colon carcinomas, *Cytometry* **38** (1999), 184–191.

[5] M.A. Chapman, J.D. Hardcastle and N.C. Armitage, Five-year prospective study of DNA tumor ploidy and colorectal cancer survival, *Cancer* **76** (1995), 383–387.

[6] M. Cosimelli, I. D'Agnano, M. Tedesco, C. D'Angelo, C. Botti, D. Giannarelli, S. Vasselli, F. Cavaliere, G. Zupi and R. Cavaliere, The role of multiploidy as unfavorable prognostic variable in colorectal cancer, *Anticancer Res.* **18** (1998), 1957–1965.

[7] W.E. Enker, M. Kimmel, E.S. Cibas, M.L. Cranor and M.R. Melamed, DNA/RNA content and proliferative fractions of colorectal carcinomas: a five-year prospective study relating flow cytometry to survival, *J. Natl. Cancer Inst.* **83** (1991), 701–707.

[8] H.L. Flyger, J.K. Larsen, H.J. Nielsen and I.J. Christensen, DNA ploidy in colorectal cancer, heterogeneity within and between tumors and relation to survival, *Cytometry* **38** (1999), 293–300.

[9] J.B. Fozard, P. Quirke and M.F. Dixon, DNA aneuploidy in ulcerative colitis, *Gut* **28** (1987), 642–644.

[10] M.C. Jacob, M. Favre and J.C. Bensa, Membrane cell permeabilization with saponin and multiparametric analysis by flow cytometry, *Cytometry* **12** (1991), 550–558.

[11] T. Kitagawa, K. Matsumoto, K. Iriyama and H. Suzuki, Prognostic significance of the bromodeoxyuridine labeling index in primary colorectal carcinoma, *Surg. Today* **27** (1997), 613–616.

[12] M. Kouri, S. Pyrhonen, J.P. Mecklin, H. Jarvinen, A. Laasonen, K. Franssila and S. Nordling, The prognostic value of DNA-ploidy in colorectal carcinoma: a prospective study, *Br. J. Cancer* **62** (1990), 976–981.

[13] M.P. Leers, B. Schutte, P.H. Theunissen, F.C. Ramaekers and M. Nap, Heat pretreatment increases resolution in DNA flow cytometry of paraffin-embedded tumor tissue, *Cytometry* **35** (1999), 260–266.

[14] M.P. Leers, P.H. Theunissen, T.B. Schutte and F.C. Ramaekers, Bivariate cytokeratin/DNA flow cytometric analysis of paraffin-embedded samples from colorectal carcinomas, *Cytometry* **21** (1995), 101–107.

[15] M.D. Linden, A.K. el Naggar, S.D. Nathanson, G. Jacobson and R.J. Zarbo, Lack of correlation between flow cytometric and immunohistologic proliferation measurements of tumors, *Mod. Pathol.* **9** (1996), 682–689.

[16] T. Mazzei, F. Tonelli, E. Mini, P. Mazzoni, A. Pesciullesi, R. Valanzano, F. Ficari, C. Biondi and P. Periti, Flow cytometric analysis of DNA ploidy and cell proliferation activity in colorectal carcinoma, *Anticancer Res.* **15** (1995), 2247–2253.

[17] H.J. Nielsen, C.S. McArdle, F. Moesgaard and the RANX05 study group, The effects of Ranitidine on long-term survival in primary colorectal cancer, *GI Cancer* **2** (1998), 227–233.

[18] R. Palmqvist, A. Oberg, C. Bergstrom, J.N. Rutegard, B. Zackrisson and R. Stenling, Systematic heterogeneity and prognostic significance of cell proliferation in colorectal cancer, *Br. J. Cancer* **77** (1998), 917–925.

[19] S.E. Petersen, M. Lorentzen and P. Bichel, A mosaic subpopulation structure of human colorectal carcinomas demonstrated by flow cytometry, *Acta Pathol. Microbiol. Scand. Sect. A* **Suppl 274** (1981), 372–376.

[20] A.E. Pinto, P. Chaves, P. Fidalgo, A.G. Oliveira, C.N. Leitao and J. Soares, Flow cytometric DNA ploidy and S-phase fraction correlate with histopathologic indicators of tumor behavior in colorectal carcinoma, *Dis. Colon Rectum* **40** (1997), 411–419.

[21] A. Salud, J.M. Porcel, B. Raikundalia, R.S. Camplejohn and N.A. Taub, Prognostic significance of DNA ploidy, S-phase fraction, and P-glycoprotein expression in colorectal cancer, *J. Surg. Oncol.* **72** (1999), 167–174.

[22] M.K. Samoszuk, G. Sallash, K. Chen, E. Carlton and T. Oldaker, Association between flow cytometric S-phase fraction and apoptotic rate in breast cancer, *Cytometry* **26** (1996), 281–285.

[23] A. Sampedro, A. Salas-Bustamante, M. Lopez-Artimez, J.L. Garcia-Muniz and G. Urdiales, Cell cycle flow cytometric analysis in the diagnosis and management of colorectal carcinoma, *Anal. Quant. Cytol. Histol.* **21** (1999), 347–352.

[24] T. Sugai, S. Nakamura, W. Habano, N. Uesugi, H. Sato, O. Funato, S. Sasou, S. Orii and C. Itoh, Analysis of subclonal expansion of colorectal carcinomas by flow cytometry, *Virchows Arch.* **434** (1999), 437–441.

[25] H. Tomoda, H. Baba, T. Saito and S. Wada, DNA index as a significant predictor of recurrence in colorectal cancer, *Dis. Colon Rectum* **41** (1998), 286–290.

[26] H. Tonouchi, K. Matsumoto, T. Kinoshita, H. Itoh and H. Suzuki, Prognostic value of DNA ploidy patterns of colorectal adenocarcinoma: univariate and multivariate analysis, *Dig. Surg.* **15** (1998), 687–692.

[27] K.S. Venkatesh, D.J. Weingart and P.J. Ramanujam, Comparison of double and single parameters in DNA analysis for staging and as a prognostic indicator in patients with colon and rectal carcinoma, *Dis. Colon Rectum* **37** (1994), 1142–1147.

[28] L.L. Vindelov and I.J. Christensen, A review of techniques and results obtained in one laboratory by an integrated system of methods designed for routine clinical flow cytometric DNA analysis, *Cytometry* **11** (1990), 753–770.

[29] L.L. Vindelov, I.J. Christensen, N. Keiding, M. Spang-Thomsen and N.I. Nissen, Long-term storage of samples for flow cytometric DNA analysis, *Cytometry* **3** (1983), 317–322.

[30] L.L. Vindelov, I.J. Christensen and N.I. Nissen, A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis, *Cytometry* **3** (1983), 323–327.

[31] L.L. Vindelov, I.J. Christensen and N.I. Nissen, Standardization of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards, *Cytometry* **3** (1983), 328–331.

[32] L. Webster, R.J. Hodgkiss and G.D. Wilson, Cell cycle distribution of hypoxia and progression of hypoxic tumour cells in vivo, *Br. J. Cancer* **77** (1998), 227–234.

[33] R.P. Wersto, R.L. Liblit, D. Deitch and L.G. Koss, Variability in DNA measurements in multiple tumor samples of human colonic carcinoma, *Cancer* **67** (1991), 106–115.

[34] R.P. Wersto and M. Stetler-Stevenson, Debris compensation of DNA histograms and its effect on S-phase analysis, *Cytometry* **20** (1995), 43–52.

[35] S. Wingren, C. Guerrieri, B. Franlund and O. Stal, Loss of cytokeratins in breast cancer cells using multiparameter DNA flow cytometry is related to both cellular factors and preparation procedure, *Analyt. Cell. Pathol.* **9** (1995), 229–233.

[36] T.E. Witzig, C.L. Loprinzi, N.J. Gonchoroff, H.M. Reiman, S.S. Cha, H.S. Wieand, J.A. Katzmann, J.K. Paulsen and C.G. Moertel, DNA ploidy and cell kinetic measurements as predictors of recurrence and survival in stages B2 and C colorectal adenocarcinoma, *Cancer* **68** (1991), 879–888.

[37] T. Yamamoto, K. Matsumoto and K. Iriyama, Prognostic significance of the DNA index in a colorectal cancer, *Surg. Today* **28** (1998), 792–796.

[38] R.J. Zarbo, R.E. Nakhleh, R.D. Brown, J.J. Kubus, C.K. Ma and P. Mackowiak, Prognostic significance of DNA ploidy and proliferation in 309 colorectal carcinomas as determined by two-color multiparametric DNA flow cytometry, *Cancer* **79** (1997), 2073–2086.

[39] R.J. Zarbo, D.W. Visscher and J.D. Crissman, Two-color multiparametric method for flow cytometric DNA analysis of carcinomas using staining for cytokeratin and leukocyte-common antigen, *Anal. Quant. Cytol. Histol.* **11** (1989), 391–402.