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MEETING REPORT

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Flow cytometric DNA and phenotype analysis in pathology

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Abstract This meeting report summarizes the presentations of three different groups that are active in the field of flow cytometry (FCM) in relation to diagnosing and classification of proliferative disorders. The report starts with the contribution from Regensburg about the developments in DNA FCM, the progression to dual parameter determinations, and combination of immunophenotyping in combination with DNA. In the second part, the use of FCM for the detection of isolated tumor cells in the peripheral blood from patients with prostate or breast cancer is discussed in a contribution from Münster. In the third part, from Heerlen, the use of multi-parameter FCM on formalin-fixed paraffin-embedded tissues from solid tumors is discussed as a new development and application in routine surgical pathology.

Keywords Flow cytometric DNA · Phenotype analysis · Pathology

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DNA analysis and dual parameter flow cytometry: selected clinical applications and the need for standardization (Gero Brockhoff and Ruth Knuechel)

DNA analysis is one of the earliest applications in flow cytometry (FCM). Already in 1969, Hudson et al. introduced propidium iodide to isolate closed circular DNA [31] and, in the same year, Dittrich et al. introduced ethidium bromide for flow-cytometric cell cycle analysis [21]. A little later, in 1973, Crissman et al. performed the first dual parametric measurements and investigated simultaneously DNA and protein content of mammalian cells [20] and, in 1977, DNA measurements with ultra violet (UV)-excitable Hoechst dyes were published [1].

Today, DNA analysis is a widespread flow-cytometric application and of particular interest both in experimental and diagnostic procedures. Fluorochromes interacting stoichiometrically with the DNA are required for quantitative ploidy and cell cycle analysis. A large number of DNA dyes are available with different properties in terms of cell permeability, DNA-base binding specificity, and excitation and emission maxima. Table 1 lists (arbitrarily) some fluorochromes suitable for DNA measurements. UV-excitable DNA dyes caught on for single-parametric measurements, while propidium iodide became the most frequently applied fluorochrome for bivariate analysis combined with immunophenotyping. The main information of interest, which is obtained from flow-cytometric DNA measurements is (1) whether or not a (sub-)population with an abnormal DNA content is present, (2) the absolute DNA content of the population of interest (expressed as DNA index DI compared to a diploid reference population), and (3) the proportional distribution of cells in different cell cycle phases, especially the amount of the S-phase fraction (SPF). Abnormal DNA content and/or increased SPFs are frequently observed in a large variety of human malignancies. However, the detection of abnormal DNA content and prolif-

Table 1 Selection of some DNA fluorochromes usable for flow-cytometric DNA analysis. *7-AAD* 7-aminoactinomycin D; *DAPI* 4′-6′-diamidino-2-phenylindole

Nucleic acid probes	Excitation (nm)	Emission (nm)	Laser line	Vital dye
Ethidium bromide	493	620	488	No
Propidium iodide	536	617	488	No
7-AAD	440	650	488	No
Chromomycin A3	445	575	457	No
Mithramycin	445	575	457	No
Acridine orange (DNA)	503	640	488	Yes
Acridine orange (RNA)	503	530	488	Yes
Hoechst 33342	343	483	UV	Yes
Hoechst 33258	345	478	UV	Yes
DAPI	345	455	UV	Yes
YOYO-1	491	509	488	No
TOTO-1, TO-PRO-1	509	533	488	No
TOTO-3, TO-PRO-3	642	661	635	No
Several SYTO dyes	500	510–550	488	Yes

eration fractions did not reveal an unambiguous value within the clinical routine diagnosis. Organ-related overviews, already compiled in 1993, demonstrated the lack of preparational and data acquisition standardization when a large number of studies were compared [50]. From an interlaboratory point of view, there is a forceful need for standardization and comparability of DNA analysis until today.

Static DNA measurements give useful information of total DNA content expressed as DI and distribution of cells in different cell cycle phases (G0/G1, S, and G2/M phase). This kind of analysis reflects a snapshot of distribution of cells in different cell cycle phases at a particular time point. However, cycling of a cell population through different cell cycle phases is a dynamic and complex controlled process, and its regulation is of special interest in many scientific and clinical fields. Tissue growth and maintenance is determined by cell growth and multiplication and cell death (via necrosis and apoptosis), whereas the increase of cell numbers within a given time is determined by cells capable of entering the cell cycle from the G0- to G1-phase, cells actively cycling (i.e., cells in G1, S, or G2/M phase), and the duration of any cell cycle phase. Static DNA analysis reveals no information about the proportion (percentage) of quiescent cells or the duration of cell cycle phases. However, dynamic cell cycle kinetics allow the evaluation of the time course of cell populations running through the cell cycle. Two different and more sophisticated flow cytometric techniques can be applied to get kinetic cell cycle information mainly depending on the instrument equipment that is available. The BrdU/Hoechst quenching technique requires 488 nm and UV excitation [43, 46], but the anti-BrdU technique alone only requires 488 nm excitation [22]. These techniques allow the simultaneous assessment of growth fraction, lag-time, compartment exit rate, compartment duration, and compartment arrest, which are indispensable information for understanding cell cycle regulation and control. We used the anti-BrdU technique in order to detect changes of the duration of cell cycle phases in the c-erbB2 receptor overexpressing breast carcinoma cell lines in response to growth factors able to bind to epidermal growth factor receptor (EGFR) or EGFR-related receptors [14]. The c-erbB2 receptor overexpressing breast tumor cells became the focus of an immunological cancer therapy with Herceptin [48]. Although it is known that this monoclonal antibody therapy has different biological and immunological effects, its mechanism of interference with cell cycle regulation is not understood [52]. We have shown that the regulation of the cell cycle differs in the c-erbB2 receptor overexpressing SK-BR-3 and BT474 breast carcinoma cells. With the help of the anti-BrdU technique, we demonstrated growth inhibition (prolongation of G1 phase) in SK-BR-3 cells and growth stimulation (shortening of G1 phase) in BT474, as response to EGF treatment. In addition, we could correlate these findings to different patterns of receptor interaction between receptors belonging to the EGFR family, investigated with the fluorescence-resonance-energy-transfer (FRET) technique [14]. The data impressively demonstrate the comprehensive and detailed information that can be obtained from multi-parametric DNA measurements combined with dynamic and functional assays.

A requirement for dual-parametric analysis of tumor cells derived from solid tumors is to preserve antigen integrity of whole cells in contrast to single parameter measurements, where isolated cell nuclei can be analyzed. Different approaches can be applied to address gentle tissue disaggregation, including enzymatic or mechanic procedures, and the combination of both [16]. The immunolabeling procedure has been verified on fixed tissue sections from normal colon epithelial tissue and colon carcinoma [32]. Cytokeratin (CK) in epithelial cells can be labeled in the cytoplasm using both directly fluoresceinated monoclonal antibody (mAb) and the indirect immunofluorescent procedure. This approach allows for an improved flow cytometric analysis, where the acquisition of the DNA data is controlled by the green fluorescence signal of the CK-positive cells.

Single cell suspensions can be prepared from fresh, frozen or paraffin-embedded material using accurate mechanical procedures. A tissue adjusted and maximized gentle tissue disaggregation is one of the first steps in flow cytometric DNA analysis, which needs to be become standardized and reproducible. In order to address this aspect, we systematically investigated 308 fresh colorectal surgical specimens from normal colonic mucosa and tumor tissues. We divided the tissue samples into aliquots for ordinary manual dissociation with tweezers and scalpel and performed quality control in comparison to an automated disaggregation method [13]. The study was performed to address the reliability of DNA measurements in terms of quality of acquired DNA histograms described by the coefficient of variation (CV) of the G0/G1 peak, the yield of CK-positive cells, the amount of cell aggregates and debris, the percentage of SPF compared with non-malignant tissue, and the identifica-

tion of small malignant subpopulations within a tumor sample. No significant differences in CV of CK-gated versus ungated cells were found. Normal colon mucosa served as a reliable internal, diploid DNA control.

Automated dissociation lead to a significantly higher gain of CK-positive cells relative to the percentage of CK-positive cells after manual tissue disaggregation. CK gating led to a clear-cut separation of SPF fractions within the respective ploidy groups, irrespective of manual or automated dissociation. The SPF increased significantly from normal tissue to diploid and non-diploid tumors. In conclusion, the automated tissue preparation with the Medimachine (Dako, Denmark) allows for a simple cell isolation for dual DNA/CK flow cytometric measurement, improving the gain of CK-positive cells and facilitates a gentle cell isolation from solid colon tissue, therefore contributing to a standardized DNA analysis.

Furthermore, with respect to software supported data handling, the development to standardized analysis is proceeding. Recently, a study was published in order to develop a unified prognostic model for node-negative breast cancer patients from flow cytometric DNA histograms [2]. The goal of this study was to identify and correct for factors that may have mitigated the usefulness of SPF in breast cancer patients, to identify possible improvements to current FCM methodology, and to demonstrate transference of the technique to other laboratories. In summary, it could be shown that when 350 data from different laboratories were analyzed following well-defined guidelines, the (1) SPF, (2) ploidy, and (3) the presence of an aneuploid cell fraction appeared as independent prognostic factors, i.e., DNA analysis can provide strong prognostic information for node-negative breast cancer patients. Thus, the evaluation of a significant number of data from node-negative cases have shown the potential for standardization [2]. In addition, detailed studies of semiautomated cell cycle analysis from more than 1000 fresh breast cancer cases demonstrated interlaboratory reproducibility of data evaluation when systematically applied [3, 4, 5].

Despite lack of standardization, flow cytometric DNA analysis has the potential of valuable diagnostic information and still is of current oncological and hemato-oncological interest. Several studies indicate the current clinical value of flow cytometric DNA measurements both for solid tumors and leukemic diseases as demonstrated by selected examples:

High-resolution DNA analysis in order to detect aneuploid and near diploid subpopulations is successfully performed in a single parametric fashion after cell nuclei isolation in head and neck cancer [28, 29]. The detection of aneuploidy was shown to be an independent prognostic parameter in squamous cell carcinomas of the oral cavity [30], and the development of aneuploidy could be found in correlation with tumor progression [45]. UV-excitable DNA dyes, such as Hoechst and DAPI (4′-6′-diamidino-2-phenylindole) are used as superb fluorochromes to generate high quality DNA histograms verified by extremely small CVs. These dyes can be utilized not only with UV laser excitation but can also be illuminated with mercury arc HBO lamp-equipped instruments well suited for routine applications [44].

DNA assessment performed in combination with immunophenotyping is successfully applied in different leukemic diseases. The DNA content emerged as a valuable additional tool to detect malignant aneuploid cells in multiple myelomas [42] and minimal residual disease after chemotherapy in acute lymphocytic leukemia (ALL) patients [41]. Aneuploid plasma cells can be identified with high sensitivity via CD19 or CD20 phenotyping, and the identification of minimal malignant subpopulations was described as superior compared with microscopic evaluation. In these studies, the flow cytometrical detection of aneuploidy served as a reliable complementing tool for diagnosis and therapy monitoring.

The analysis of tumor cells derived from heterogeneous solid tissues has to take into account that in fact not only the true tumor cell population is examined but also many other cell types (lymphocytes, macrophages, stromal cells, etc.) contribute to the final result. In parallel with the DNA staining, an additional immunophenotyping can help to identify the (tumor) cells of interest. In case of epithelial tumors, CK is mostly used as a discriminating parameter in order to exclude CK-negative stromal and inflammatory cells from the flow-cytometric analysis. In the case of gastro-intestinal carcinomas or adenocarcinomas, good results in terms of tumor cell specificity have been acquired using a mAb specific for CKs 8, 18, and 19, according to the Moll classification [38, 39]. Furthermore, antibodies directed against different CK subtypes have been successfully applied in trivariate flow analysis of lung of cancer specimens to separate estimation of ploidy status and cell cycle parameters separately in phenotypically heterogeneous malignancies [33].

A powerful extension of dual-parameter measurements for cell selection and DNA analysis is the inclusion of additional markers as, e.g., proliferation markers [47], tumor-suppressor genes or oncogenes [12], or other relevant antigens. Today, three- or four-parameter analysis is reliably possible with routine flow cytometers with single laser [12, 17, 18] or dual laser excitation [19]. Valuable, comparable information regarding antigen densities can be expected from absolute quantification methods [10, 11, 12, 14, 15].

Blood-borne cancer cells in the vascular system; a challenge for FCM (Burkhard Brandt, Hartmut Schmidt, Gabriela De Angelis, Elke Eltze, Axel Semjonow)

Tumor diagnostic investigations on the peripheral blood of patients in today's clinical laboratories are based mainly upon the demonstration of proteins synthesized either by tumor cells or by healthy tissue as a reaction to malignant growth (tumor markers). A number of these "tumor markers" have now become useful parameters

for tracking the progress of clinical disease. Due to lacking specificity, however, a preparative staging or an individually customized therapy cannot be geared toward information provided by the tumor markers. For this reason, in addition to serological confirmation, attempts have been made to confirm metastasizing tumor cells themselves in the blood of patients so that a parameter can be found for estimating a patient's metastatic risk or the success of therapy.

The first reports on the demonstration of tumor cells in peripheral blood go back to the start of the last century. The experimental papers of Zeidman, Fidler, and Gullino laid the foundations for the demonstration of tumor cells in peripheral blood [23]. Zeidman concluded from experiments on rabbits that tumor cells can traverse capillaries and spread to the whole organism. Fidler was the first to measure blood-borne metastasizing tumor cells quantitatively in the peripheral blood of experimental animals. Gullino et al. calculated that the daily cellular turnover of a tumor is very high in relation to the tumor mass. In that model, they also found that a malignant tumor can induce metastases both during times of progression and regression. The absolute number of tumor cells in arterial blood was 1/12 of that present in the veins of the upper leg and, in this way, tended to correspond to the normal distribution of the blood volume. The studies presented verified that the released tumor cells distribute so rapidly in the vascular system that blood obtained by venous puncture can be employed for detecting tumor cells in peripheral blood. Following this assumption, it has now become possible to demonstrate tumor cells in peripheral blood indirectly using the reverse transcription polymerase chain reaction (RT-PCR), which reverse transcribes messenger (m)RNA produced by tissue-specific genes [40].

FCM allows the simultaneous measurement of fluorescence and scattered light signals from single cells. Several thousand cells can be examined in a relatively short time. For this reason, it seemed reasonable to apply this method for demonstrating circulating tumor cells. Hamdy et al. were the first to show prostate-specific antigen (PSA) positive cells in the peripheral blood of prostatic carcinoma patients using FCM [25]. The large proportion of patients showing a positive cell response and the large number of cells found in patients' blood allow us to assume that the method also registers cells other than carcinoma cells. We then also found PSA-positive cells in patients' blood, which carried the pan-leukocyte CD45 marker [7]. The cells were isolated with a cell sorter, and the RNA was analyzed using a sensitive RT-PCR on PSA and actin (positive control). No PSA mRNA could be measured in the cells, which indicated that a PSA-internalization phenomenon was taking place. We also analyzed cells from peripheral blood using fluorochrome-conjugated antibodies against PSA and the monocyte marker CD14 using FCM. The PSA–CD14 double positive cells found appeared to indicate a group of patients having a lower metastatic risk [6]. Newer immunotherapeutic experimental approaches showed that PSA peptides are able to bring about a specific T lymphocyte response in prostatic carcinoma patients. It can therefore be assumed that processed PSA, e.g., by antigen-presenting cells (CD14 positive monocytes), induces a specific immune response and can in this way reduce the risk of metastasis. In another study, we showed that prostatic carcimoma cells could be isolated with the combined method of density gradient centrifugation and immunomagnetic separation, and the use of epithelial specific (CK) and PSA-specific antibodies. Using this procedure, it was found that the number of cells rises with increasing tumor stage and capsule infringement of the tumor [7]. From this, we have developed a concept for characterizing the cells via demonstration of genomic alterations; this should produce the first results next year. The genome of the cells will be investigated using a procedure for indirectly detecting alterations of defined gene locations, i.e., multiplex microsatellite PCR. Identified cellular genomic alterations will be compared with the alteration profile of the primary tumor and assigned according to the clinical stage and clinical progress of the patients.

From both a clinical and biological perspective, there are a range of similarities between prostatic and mammary carcinomas. At the time when the tumor is first diagnosed and primary therapy is started, a non-localizable distant metastasis (occult metastasis) is present in about 20% of cases despite the presence of otherwise favorable prognostic parameters, such as a negative lymph node status. The presence of distant metastasis is a decisive factor for determining the success of primary and adjuvant therapy. An improved preoperative staging would improve the success rate of operative therapy and would allow patients with a raised metastatic risk to be directed to a systemic therapy in good time. The most frequent site of metastasis for both carcinomas is the skeleton and, for this reason, strategies to develop prognostic markers by isolating and characterizing epithelial cells from peripheral blood are also meaningful for mammary carcinomas. Considering this, we also applied our method to mammary carcinomas. Here it could be shown that c-erbB-2-expressing cells correlate positively with the tumor stage [8]. Adjuvant therapy with an anthracyclin-containing regimen resulted in a reduction in cells [9]. Discontinuation of therapy led to an increase in cells combined with the demonstration of distant metastasis. The extent to which the preoperative demonstration of c-erbB-2-positive cells in peripheral blood can allow a prediction of the overall metastatic risk requires further studies on larger patient collectives. It is highly likely, however, that such tumor cell populations are critical for determining the risk of metastasis. This study leads us to demand that thorough searches for metastases be performed postoperatively and at short intervals in such high-risk patients and that if the situation should arise, an aggressive chemotherapy be started in good time. In addition, the possibility has arisen to control novel therapeutic approaches for mammary carcinomas, e.g., herceptin therapy.

The use of multiparameter (MP)FCM on formalin-fixed, paraffin-embedded tissue: a new tool for daily routine in surgical pathology (Marius Nap, Mathie P.G. Leers)

Histo- and cytopathology traditionally combine the morphological characteristics of cells and tissues with knowledge about mechanisms of disease. In most cases, this approach results in a diagnostic classification of the specimen that was examined. In specific situations, the clinical information can be integrated in this classification, which results in individualized treatment and follow-up. Identification of irregularities in or aberrations from the normal configuration of cells can be supported by special stains, such as immunohistology and molecular biological techniques. However, despite the high technical level of these tools, their application leads to qualitative results, and the determination of a quantitative interrelationship of the various components in tissue sections or cytological preparations still remains a task with a high level of subjective influences. To some extent, image analysis, either based on gray values or color, can be of help in the quantification of tissue components, such as blood vessel diameter [24, 51, 54]. Although cytological specimens will allow the observation of complete cells, histological sections will only show fractions of the cell nucleus and cytoplasm and their membranes because of the limited thickness of tissue sections. In addition, the presence of interstitial substances of various origin can hamper a correct interpretation of the image. The above-mentioned aspects lead us to the conclusion that in a situation where quantitative interpretations of cell characteristics are important, a different approach should be looked for. Whereas immunohistology and related techniques primarily deal with qualitative identification of cell components, FCM is focussed on the quantification of the number of events or cells that pass a laser beam. In addition, phenotypic properties of cells can be identified using immunological labeling techniques using different fluorochrome labels with non-overlapping wavelengths, allowing simultaneous analysis of various cell characteristics. Simultaneously, information on the DI and the place of the cell in the cell cycle can be obtained and related to changes in shape and antigenic phenotype of the cells.

If cytological specimens are involved, cells are already separated and can be prepared and submitted for FCM without additional isolation techniques. For the examination of tissues, a proper separation step should be included before FCM analysis can be performed. Fresh or frozen tissues are usually treated by mechanical devices for single-cell isolation. Formalin-fixed, paraffin-embedded specimens can be treated either by mechanical or enzymatic treatment, but both methods result in high amounts of cell fragments, loss of antigenic epitopes, and poor quality of the DNA histogram. Because the use of formalin-fixed, paraffin-embedded tissue has many logistic advantages for a possible application of FCM in surgical pathology, we looked for an alternative approach. The use of high temperature treatment of 50-µm sections of the paraffin-embedded tissue block for several hours in an acidic environment appeared to be an excellent pretreatment both for antigen retrieval after formalin fixation and for the preparation of a cell suspension by a very short enzymatic digestion step of only 10 min. Comparison of this procedure with the conventional “long” enzymatic digestion procedure [26] resulted in doubling of cell recovery and minimization of background and debris. In addition, when the suspension was incubated with antibodies against the CK filaments, the mean fluorescence intensity increased, probably due to more intact cells showing a more extensive availability of the cell skeleton. It also appeared that the DI was more constant within duplicate measurements and, on top of that, was not influenced by the time of exposure to fixatives, such as formalin [35].

Due to the development of the above-mentioned alternative method for the preparation of single-cell suspensions from paraffin-embedded tissues, routinely processed material, and thus also archival blocks, can be used almost without restrictions for FCM, where both DNA analysis and immunophenotyping can be performed.

Until now, the use of FCM in surgical pathology as part of the daily routine has largely been restricted to either DNA analysis or immunophenotyping as separate procedures for solid tumors or hematological neoplasms, respectively. The combination of DNA information with immunophenotyping into MPFCM allows an objective registration of positive fluorescence signals due to specific binding of one or more antibodies in combination with the signal obtained by the DNA-binding fluorochromes. For routine MPFCM, we now use the GALAXY FCM from Dako in combination with a variety of mono- and polyclonal antibodies.

The application of this technique in routine surgical pathology can primarily be found in those cases where immunophenotyping is normally associated with semiquantification of subpopulations of cells in immunohistochemistry. Using dedicated software, the data from subpopulations of cells that has been collected in the computer can be analyzed in various combinations of both DI and immunophenotype using so-called gating procedures. Looking for areas where this technique could contribute to the quality of histo- and cytopathology in daily routine, we identified at least two fields of interest. One deals with the determination of clonality of light chain in lymphoid proliferations, and another deals with the presence or absence of steroid hormone receptors and other characteristics of biological parameters in breast cancer [36]. In both situations, the combination of a qualitative (the specific immunoreaction) aspect and a quantitative aspect (the size of the fraction of cells with a specific phenotype) has to be made within the relevant subpopulation of cells (either CD79a-positive B lymphocytes or CK-positive epithelial cells) in the specimen. In lymphoid proliferations, this method allows for an objective evaluation of the kappa/lambda ratio within the

B-cell population either below 0.5, above 3, or in between these two values to support a choice for mono or polyclonality [37, 55]. In lymphoid proliferations, the determination of surface or cytoplasmic immunoglobulins using immunohistochemistry is often obscured by the presence of these proteins in the interstitial fluid. The MPFCM approach enables one to observe the cells separated from the interstitial substances and, therefore, it becomes possible to calculate the ratio of kappa or lambda light chains on lymphocytes. In view of the new classification of malignant lymphomas in small cell and large cell tumors, MPFCM can also contribute by using the forward scatter information that deals with the size of the measured events.

In breast cancer, the information on estrogen receptors' and progesterone receptors' expression cannot only be provided for the entire fraction of epithelial tumor cells, but also separately for the diploid and aneuploid epithelial fractions within the same tumor. After having analyzed now more than 230 different primary breast tumors, it appears that the average diploid fraction in real aneuploid tumors is approximately 40% of the tumor and that these diploid tumor cells show almost the same percentage of receptor positivity as observed in purely diploid tumors. However, the aneuploid subpopulation within the same tumor showed a significant loss of receptor protein expression (unpublished observations). We expect that the combination of this information with the clinical course of the tumor will eventually result in a more precise classification of possible sensitivity for hormonal treatment.

A major advantage of this MPFCM approach is that morphological information is still available since the block from which the 50-µm thick sections are taken is also used for production of routine hematoxylin and eosin staining. The admixture of non-tumor elements can thus be anticipated and, in special cases, a preselection of the area of interest can be done even before the cell suspension preparation step.

Another aspect that comes for free with the MPFCM is the possibility of calculating the SPF of the tumor cells. As long as sufficient cells are available in the subfractions, with a minimum of 5000 cells [27, 49, 50], the percentage of cells in S phase of the cell cycle can give an indication of the proliferative activity of the different subpopulations which compose the tumor.

From the experience obtained so far, there appears to be a strong correlation between the loss of a diploid DNA content and elevation of the SPF, and there is no adverse effect on the accuracy of the determination by formalin fixation. Due to the fact that a selection of epithelial cells from the total population has been made, the SPF appear to be slightly higher and more specific related to tumor activity because the dilution effect of stromal components is ruled out.

The two fields of application for MPFCM mentioned above are by no means limiting. Because cell membrane integrity is relatively well-preserved with this approach, and epitope retrieval of the formalin-fixed tissue is realized by the heating process, other aspects that deal with tumor biology can be analyzed.

One of these aspects is the percentage of cells expressing E-cadherin, a cell adhesion molecule, or the quantitative fraction of Her-2/neu positive tumor cells. The first appears to be inversely related with the presence of lymph node metastasis [53] and the second, apart from being associated with the presence of lymph node metastasis, will become an important parameter in the selection of patients for antibody-mediated tumor therapy [48, 52]. At present, we are evaluating the MPFCM determination of Her2/neu in comparison with the already accepted immunohistological classification protocol. By the end of this year, we hope to have a good impression of possible discrepancies between the two methods because of a possible false-positive interpretation of cytoplasmic staining in MPFCM. The combination of DI and SPF with Her2/neu expression will most likely be of additional value in this process. Meaningful expression of Her2/neu on the cell membrane should logically be associated with increased SPF and aneuploid DI. This might also be of help in the evaluation of antibody reactivity with the functional receptor. Furthermore, from the work of Rupa et.al. (unpublished observations) in colon carcinomas, MPFCM appears to be suitable for calculating the size of the apoptotic fraction in epithelial tumors using a combination of pan CK antibodies and the M30-cytodeath antibody, recognizing a neo-epitope on CK 18 that is induced by caspase cleavage of this molecule early in the apoptotic cascade [34]. The fraction of apoptotic cells appears to overrule the prognostic meaning of the well-known Duke's classification in colon cancer in a 10-year survival period.

Recently, we have also applied CK/DNA MPFCM for the analysis of sentinel lymph nodes in breast cancer. To our surprise, we could demonstrate that MPFCM was more sensitive than either multilevel histology or immunohistochemistry in the analysis of 238 lymph nodes. In addition, it appeared that the majority of micrometastases, smaller than 2 mm, presented with a diploid DNA content, irrespective of the DNA profile of the primary tumor (unpublished observations). We wonder how this way of analysis of metastatic tumor cells can contribute to our knowledge on metastatic and survival potential of tumor cells. Based on our present experience, which is still limited, we are convinced of the potential contribution of MPFCM in the further development of tumor characterization in surgical pathology, and many applications will follow this preliminary list.

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