Journal of Cellular Biochemistry 17G:98–106 (1993)

Quantitative Microscopical and Confocal Laser Scanning Microscopy for Intermediate Endpoint Biomarkers in Breast Cancer: Potential and Reproducibility

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Abstract Diagnostic quantitative pathological (QP) determinations are increasingly used in our hospital. The number of requests for QP for reference materials is rising rapidly. This is understandable; quantitative assessments have a strong prognostic value and can be very reproducible, depending on the care taken with a number of factors including cell and tissue processing, application of the appropriate stains, and the measurement protocol used. As to the latter, systematic random sampling gives the best intra- and interobserver agreement (with correlation coefficients between observers for certain features ≥0.94).

Flow cytometric determinations are often regarded as more reproducible than interactive morphometry due to the high speed of the assessments, the large number of objects measured per specimen, and the lack of observer interaction. Indeed, flow cytometrically assessed DNA ploidy is very reproducible, even though the % S-phase fraction is much more variable. Unlike image cytometry (ICM), visual inspection of cells is not easily accomplished with flow cytometry (FCM). With ICM, the fully automated measurement of DNA in thousands of cells is possible in 3–5 minutes, with a very low coefficient of variation (≤2% for the diploid and tetraploid peak of liver cell nuclei). ICM also allows measurement of texture features. However, quantitative immunohisto/cytochemical determinations may not always be as reproducible as sometimes believed. Recently, we found large variations in the measurements, made by a commercially available image processing instrument, of the estrogen and progesterone receptors, Ki-67, cathepsin D, and *neu* protein overexpression in breast cancer.

Confocal laser scanning microscopy (CLSM) is a powerful new tool that can be used for 3-dimensional (3-D) microscopy and detects weakly fluorescent substances. We found CLSM useful to assess multidrug resistance (MDR) in tumor cells. The CLSM MDR method requires less than 50 cells, takes approximately 30 minutes, and currently is one of the most sensitive methods for assessing MDR. Moreover, 3-D CLSM of thick (50 μm) breast cancer sections can result in realistic 3-D views of whole tissue and also in accurate measurements of volume and shape factors of individual nuclei. As these features have proven to be strong prognostic factors in tumors of different sites, CLSM in combination with digital image processing (DIP) is a promising tool to accurately assess biomarkers in breast cancer patients. Cost-benefit analysis shows that morphometry, stereology, and ICM have the best price-performance ratios.

Key words: Breast cancer, intermediate endpoint biomarkers, quantitative microscopy

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Overwhelming evidence indicates that quantitative cell and tissue features have essential and strong prognostic value in a variety of different tumors [1]. Basically, two different methods are used to describe geometric cell and tissue parameters in quantitative pathology and morphometry. In breast cancer, morphometric features such as the mitotic activity index (MAI), mean nuclear area (MNA), mean nuclear volume (mean nuclear v_V), syntactic structure analysis (SSA), and cytometric characteristics such as DNA ploidy, percentage S-phase cells (% S-phase), overexpression of estrogen and progesterone receptors (ER, PR), epidermal growth factor receptor (EGFR), *neu*, and others have been associated with prognosis, and thus may be used as biomarkers.

Using these features in diagnostic and therapeutic decision making depends on additional, independent prognostic value (as shown in retrospective, and may yet be shown in prospective, independent studies), reliability (reproducibility, accuracy, bias), costs, price/performance ratio, and the availability of quality control/assurance protocols. Fleege *et al.* [2] present a detailed treatise on reproducibility and coping with different influencing factors. This paper will discuss reproducibility of morphometric and cytometric assessments. Some new applications of digital image processing (DIP) and confocal laser scanning microscopy (CLSM) will be discussed as well. Finally, an approximate price/performance evaluation of each technique will be given.

REPRODUCIBILITY OF MORPHOMETRIC ASSESSMENTS

The MAI, MNA, volume weighted mean nuclear volume v_V (*e.g.*, in breast, melanoma, cervix), and stereological features such as volume percentage epithelium (*e.g.*, ovary), and surface and length densities of glands and lumen (*e.g.*, in endometrium, gastric mucosa) are morphometric features with strong and independent prognostic values.

The MAI is very reproducible, as shown in a prospective multicenter evaluation [3]. Delayed fixation does not significantly alter mitotic rates of most tumors [4]. The influence of delay in fixation, air-drying, acidity of 10% formalin (4% buffered formaldehyde), Bouin and mercury-formalin fixatives, acetone and ethanol dehydration, and under- and overstretching of the paraffin sections have been studied for nuclear morphometric features. Acidity had the strongest influence on nuclear area. For pH < 3, the nuclear area is approximately 25% less compared with a pH between 5 and 9. No significant differences in nuclear area were detected for a pH between 5 and 9. Differences between the nuclear area in tissue slices fixed in buffered formaldehyde, Bouin's fluid, and mercury-formalin strongly suggest that low acidity of the latter two fixatives is the vital factor. Short dehydration with acetone instead of alcohol does not influence nuclear area, but hardens the tissue and may result in lower-quality sections. Therefore, for morphometric assessments, at least one representative slice of each tumor (max. 5 mm thick) must be fixed for at least six hours in an excess of buffered 4% formaldehyde with a controlled pH of 5–9 (temperature 18°C) [5]. For immunohisto/cytochemical studies, other fixatives may be preferable.

Barry and Sharkey [6] and Chan *et al.* [7] described considerable variation in morphometric assessments with respect to measuring system parameters, in direct contrast to the data of Dardick and Caldwell [8] and our own results [1]. The method of measurement in these studies differed from ours; we therefore carried out further studies on tracing speed, "projected" particle size (total magnification at the digitizing tablet level), orientation and localization on the tablet, pen-photograph versus cursor-microscope, and particle shape [9]. Magnification, tracing tool, and tracing speed appeared to be the important factors. For a minimal particle diameter of ≥15 mm, the coefficient of variation (CV) remains stable, between 1.0–1.5%. For measurements of cancer nuclei (average diameter 6–12 μ), a total magnification at the digitizing tablet level of 2000× is usually sufficient. Interestingly, in the study by Barry and Sharkey [6], the diameters of the objects measured were too small (3–6 mm).

The reliability of measurements also depends on the selection method, sample size, measurement protocol, and different biological phenomena within the selected measurement field. These phenomena include: *object variation*, the variation of a feature from object to object (pleomorphism, anisokaryosis); *object clustering*, the presence of groups each containing objects that show a relatively high similarity for a certain feature, but with differences for the particular feature among

the groups; and *object gradient*, a steady, direction-dependent shift in the magnitude of a feature. A high degree of object variation means more objects must be measured to achieve a given level of precision. More pronounced object clustering or the presence of gradients demands that the selection method must cover the measurement field uniformly.

Within a certain area, objects measured must be selected both systematically and at random to get the best reproducible results. We recently found mean nuclear v_V very reproducible and strongly correlated to MNA in breast cancer [10]; the mean nuclear v_V was assessed both in the most atypical area (AREA) (selected on morphological criteria) and in the whole tumor section (TOTAL). With bivariate correlation analysis, the two sampling methods showed good correlation for the mean nuclear v_V values (range of the correlation coefficient: 0.92–0.97). There were no systematic intraobserver differences between the different sampling methods. The results of Observer 1 were higher with both the selective and random systematic sampling method. However, these systematic interobserver differences were small (<9% of the average value of mean nuclear v_V), much smaller than the variation between the tumors, which was over 60%.

The time required for assessments in the AREA was less than that for the determinations in the TOTAL (average: 10 versus 20 minutes), despite the similar sample size. This is understandable; in a sclerotic tumor, many microscopic fields do not contain cancer nuclei. In invasive breast cancer, assessments in the whole tumor section can be used if delineation of the measurement area cannot be easily accomplished. In small areas with a limited number of nuclei (*e.g.*, microinvasive parts), MNA can be easier to assess than mean nuclear v_V.

CYTOMETRIC ASSESSMENTS

Both flow and image cytometers are used. Flow cytometers are widely used for phenotyping and other measurements (mainly DNA ploidy). DNA ploidy assessment is very reproducible. *Specimen sampling* is especially important in DNA flow cytometry (FCM), since intratumor ploidy variations may be present. Several clones of cells with different DNA content may occur within lesions, and invasive areas may have ploidy levels different from *in situ* areas.

Tissue processing is a critical step in preparing single-cell suspensions for DNA cytometry. In DNA histograms of fresh frozen material, the CV of the diploid peak is usually lower than that in paraffin-embedded tissue [11], facilitating detection of near-diploid peaks. Though fresh frozen material is preferable, paraffin-embedded material still provides a sound alternative; Kallioniemi [12] describes correlation coefficients of 0.98 between DNA indices and 0.79 between % S-phase fraction values obtained from a large series of fresh and paraffin-embedded tissue. For paraffin-embedded material, it is advisable to use only buffered formaldehyde-fixed tissue; fixatives such as Bouin's and Zenker's fluids may disrupt nuclear integrity, resulting in inadequate histograms.

Calibration of peaks in DNA flow histograms is often done by admixing standard cells of known DNA content with tumor cell suspension (before staining). Human leukocytes can only be used as an internal standard for fresh frozen material, since the diploid peak in single-cell suspensions of fixed tissue can vary considerably.

DNA image cytometry (ICM) of Feulgen-stained specimens is not very sensitive to *tissue processing*, as long as the variations are within normal limits [13]. However, *measuring system parameters* vary for different types of image cytometers and can strongly influence the results [14].

Although in principle DNA measurements are objective, subjective elements may be introduced when interpreting histograms. The usual classification into diploid, polyploid, aneuploid, (peri)-tetraploid, or multiploid is fairly reproducible when clear-cut rules are used [15]. However, overlooking a small diploid peak next to a large near-diploid peak and misinterpreting tetraploid peaks are well-known pitfalls.

The % S-phase fraction has particular prognostic value in many tumors. Reliable results may be obtained for diploid histograms, but aneuploid histograms can be difficult. For DNA ICM histograms, the percentage of cells above a certain DNA content is usually calculated. This so-called exceeding rate (*e.g.*, 2.5c or 5c exceeding rate) may have prognostic value. In endometrial carcinoma, a 5c exceeding rate above 1.7% was the strongest unfavorable prognosticator. A DNA

ICM histogram usually contains data from only 100–200 nuclei, so different values for one or two cells can give a different prognostic classification. Subjectivity cannot be completely excluded and ambiguities and shifts can occur.

DNA FLOW AND IMAGE CYTOMETRY: BOTH HAVE THEIR (DIS)ADVANTAGES

FCM is quick and has a low CV, but it is "blind": direct visual identification of cells during measurement is not possible, and it is hard to determine whether a diploid peak in a DNA flow histogram consists of tumor or non-malignant cells. This is not usually relevant since diploid nuclei serve as a reference; however, false-negative diploid histograms may be obtained when the aneuploid cells are overshadowed by large numbers of diploid tumor or non-malignant cells, especially in non-selective DNA FCM. Oud *et al.* [16] showed that removal of the non-epithelial component changed the DNA histogram from diploid to aneuploid.

Unlike FCM, DNA ICM in tissue sections allows visual identification of cells. However, a disadvantage is the low speed of most commercially available systems, which are not fully automated; sample size of such image cytometers is restricted in practice to 100–250 cells. In thin sections (4–6 μ), 25–90% of nuclei are not present in their entirety because portions are cut off ("capping"). Thick sections may help, but often have serious overlapping of nuclei; selection of nuclei may be very slow as a result. Also, this method carries the risk that the selected nuclei are special clones, as they must have large amounts of cytoplasm. Three-dimensional (3-D) DNA cytometry in tissue sections by means of CLSM may help overcome this problem.

van Diest *et al.* [17] described a rapid, fully automated system for DNA image assessments in monolayer cytologic slides of cell suspensions. The system, which is commercially available [Pathology Image Processing Environment (PIPE), SIG™ Services, Utrecht, The Netherlands], measures 1000–2000 cells in 2–3 minutes, and the CV of the diploid and tetraploid peaks of liver nuclei in imprints is <2%. The system digitally stores each object measured, together with the x and y coordinates on the slide. All objects are displayed as a composite image; the system allows zooming in and out, and deletion of each object in the composite image. In the histogram, each bar can be indicated and all objects in that bar then displayed. Relocation of each object on the microscope is also possible to the nearest 0.5 μ.

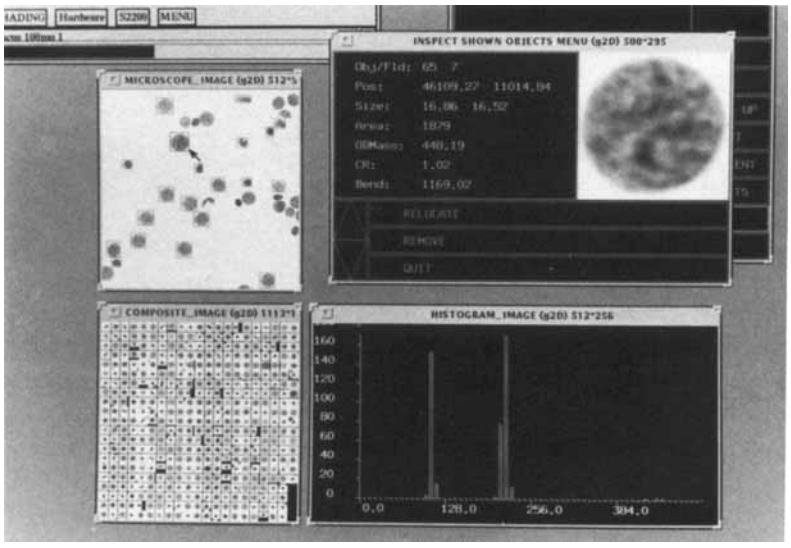

Fig. 1. Example of DNA cytometry with fully automated PIPE image digitally stored and can be magnified or relocated. The CV is very low.

Although some malignant cell nuclei have a normal DNA content [18], the chromatin pattern (morphological distribution of DNA) in normal and (pre)malignant nuclei is often clearly different. Quantitative chromatin pattern analysis measures subjectively observable differences, and also detects changes invisible to the human eye [19,20]. When correlated with DNA replication, many factors such as ion and carcinogen concentrations have strong effects on the nuclear chromatin pattern, and can thus be a more sensitive reflection of the functional state of the nucleus than total DNA content [21]. However, quantitative nuclear chromatin pattern features are also very sensitive to variations in cell and tissue processing and to measuring systems [22].

The presence of ERs in breast cancer is usually regarded as an indicator of cellular differentiation [23] and correlates with the reaction of exogenous hormones. Histological techniques, especially the immunoperoxidase techniques with monoclonal antibodies for ER determination [24,25], have opened new perspectives [26], but are inherently subjective. Attempts have been made to perform measurements by FCM, but this does not always permit verification of cell types actually labeled. This problem can be overcome by measuring the reaction intensity of ER-immunocytochemical reaction by ICM. Bacus *et al.* [27] described a quantitative evaluation by biochemical, immunohistochemical, and automated computer-assisted image analysis. Immunohistochemical evaluation incorporated both intensity and distribution of staining. An objective quantitation by computer-assisted image analysis yielded a quantitative immunocytochemical score. Comparison of this method with biochemical and immunohistochemical analysis of tissues revealed excellent sensitivities and specificities. These data indicate that automated image analysis provides an effective quantitative means of evaluating ER content of human breast cancers. Likewise, the immunocytochemical measurement of PRs, nuclear antigens in proliferating cells, cathepsin, multi-drug resistance (MDR)-associated glycoproteins, EGFR, *neu* oncoprotein, and other immunocytochemical products can be demonstrated.

Unfortunately, reproducibility of these measurements by different observers is not always perfect (Table I).

FURTHER AUTOMATION BY DIGITAL IMAGE PROCESSING

The PIPE cytometry system mentioned above also offers the possibility of automatically prescreening mitotic figures in Feulgen-stained

TABLE I. Interobserver Reproducibility of Different Morphometric PRODIT 5.1 (MNA, MAI, mean nuclear v_V) and Cytometric CAS 200/486 Features (Correlation Coefficients)

Feature	Observers 1–2	Observers 1–3	Observers 2–3
MNA	0.87	0.82	0.89
MAI	0.97	0.87	0.93
Mean nuclear v_V	0.93	0.91	0.86
DNA index imprint	0.80	0.76	0.86
DNA index section	0.46	0.76	0.66
ER	0.76	0.82	0.75
PR	0.77	0.73	0.88
Ki-67	0.60	0.63	0.76
neu	0.87	0.72	0.61
Cathepsin D	0.89	0.69	0.93
EGFR	0.91	0.69	0.93

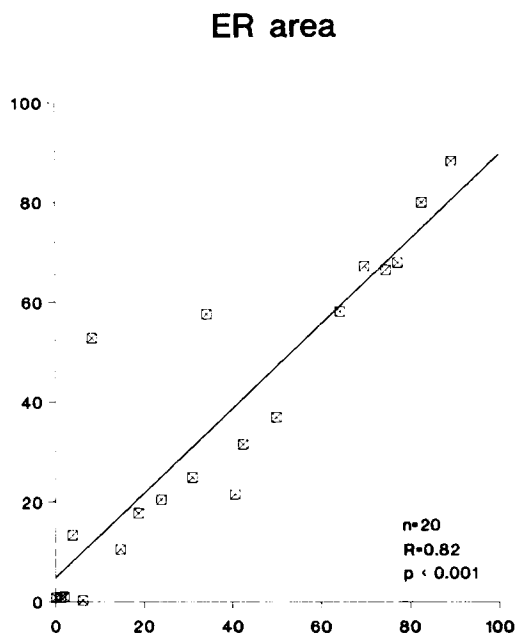

Fig. 2. Reproducibility of ER by two observers with the CAS-200 system (observers 1 and 3 of Table I).

breast cancer sections [28] and micronuclei in smear preparations. This is important since interactive assessments can be tedious, especially for micronuclei. Sample sizes should be as high as 10,000–30,000 cells to obtain statistically reliable results. Such a non-automatic count could take up to 30 hours, which is impractical. The automated method, using an interactive evaluation step, gave an accurate reflection of the mitotic count, with an almost-perfect correlation of the results with the interactive morphometry ($r = 0.998$). Therefore, this semiautomated method may be useful as a prescreening device.

The PIPE system further allows measurement of the percentage epithelium in standard paraffin-embedded tissue sections of breast premalignant and malignant lesions. The percentage of epithelium, as automatically estimated by this technique, was compared with the epithelial percentage estimated interactively. The correlation coefficient was 0.98 ($p < 0.001$), and the slope of the best linear fit ranged between 1.04 and 1.07 [29]. The image analysis technique describes results in the most epithelium-rich areas of the specimen, and may therefore be used to select these areas automatically before estimating the percentage of epithelium.

CONFOCAL LASER SCANNING MICROSCOPY

CLSM is an important new imaging tool which allows elimination of out-of-focus images, as well as the detection and visualization of weakly fluorescent particles. CLSM thus makes it possible to make serial optical slices of objects, such as nuclei, in tissue sections. By focusing up and down at high magnification, this two-dimensional (2-D) image data can be used to create 3-D reconstructions of cellular characteristics (*e.g.*, nuclear molding in certain lobular invasive breast carcinomas, deep nuclear indentations in certain lymphomas, *etc.*). Application of CLSM and such new imaging algorithms as volumetric rendering generate high quality images which give a realistic impression of the original object. Detailed 3-D visualization of tissue structures and their possible quantification may provide the pathologist with additional information, which can be helpful or even essential in making a diagnosis (*e.g.*, the volume, shape, and DNA content of individual nuclei in a breast cancer specimen) [30].

CLSM has also been used to quantitatively assess MDR. For example, changes in intracellular drug localization accompany doxorubicin resistance in MDR cells. In a study by de Lange *et al.* [31], tumor cells were incubated with the fluorescent anthracycline doxorubicin (excitation at 480 nm; emission maximum at 560–590 nm) and quantified using CLSM. The fluorescent mode was used to record the intracellular drug distribution; the absorption mode was used to define the nuclear and cytoplasmic boundaries. The cell compartments were delineated interactively on an image processing system and the ratio of nuclear fluorescence/cytoplasmic fluorescence (N/C ratio) was determined. N/C ratios were as follows: 1.8 in the Chinese hamster ovarian cell line AUXB1 and 0.1 in its MDR subline CH^RC5; 3.8 in the human squamous lung cancer cell line SW-1573 and 1.8 and 0.4 in its MDR sublines SW-1573/2R120 and SW-1573/2R160, respectively; and 3.6 in the human myeloma cell line 8226/S and 2.1 and 1.0 in its MDR sublines 8226/Dox4 and 8226/Dox40, respectively. The doxorubicin distribution was independent of the doxorubicin concentration within a range of 1–32 μM. Furthermore, the progressive mean of the nuclear/cytoplasmic doxorubicin fluores-

TABLE II. Approximate Costs in $US and Price/Performance Ratio (P/P) of the Different Quantitative Pathological Techniques

Technique	$US (in thousands)			Performance	P/P
	Equipment[1]	Workforce[2]	Overall costs[2]		
Morphometry stereology	5–10 (6)	2	8	very good	excellent
DNA image cytometry[3]	8–25 (13)	5	18	good	very good
DNA flow cytometry	10–50 (20)	4[4]	24	good	good
	30–50 (15)	3[5]	18	good	very good
CLSM[6]	50	15	65	good	n.a.
MMS[7]	10	n.a.[9]	—	—	n.a.
AI[8]	5	n.a.	—	—	n.a.

[1] per year $US, 5 year write off (median)
[2] personnel and consumables including administration, per 100 determinations; median write off, three years
[3] 1000 cells per specimen
[4] for fresh material
[5] for paraffin-retrieved material
[6] CLSM = confocal laser scan microscopy
[7] MMS = multimedia systems
[8] AI = artificial intelligence
[9] n.a. = not applicable; research tools only

cence ratios showed that a minimal sample size of 30 cells is necessary for reliable results. The results of two independent assessments showed a high reproducibility (r = 0.97). Thus, it is possible to detect relatively low levels of doxorubicin resistance by using CLSM.

COST-BENEFIT ANALYSIS

The factors influencing reproducibility of quantitative cell and tissue features have become obvious over the past decade. A number of laboratories use DNA cytometry as a routine clinical tool, and to a lesser degree, the same holds true for morphometric and stereologic analyses. This brings us to the question of benefits and costs for each method. In chapter 18 of the *Manual of Quantitative Pathology in Cancer Diagnosis and Prognosis*, we have summarized the different methods with respect to their independent or additional value [1]. Obviously, at the moment CLSM, multimedia systems, and artificial intelligence are research tools rather than clinically applied methods. Table II summarizes the approximate performance and costs of the different techniques. Space for detailed arguments is lacking here, but the data provided are based on 15 years of experience with diagnostic quantitative pathology. In our department, all methods are routinely used; however, in these economically difficult times for health care, a tendency exists to concentrate on morphometry, stereology, and DNA ICM. Costs for other ICM applications (ER, PR, Ki67, *etc.*) and a multitude of DNA ICM techniques are not further discussed here, especially since neither their reproducibility, nor their prognostic power relative to the morphometric features in breast cancer are very good (fig. 2, Table I).

ACKNOWLEDGEMENTS

The work presented is supported in part by grants 28/1814 and 28/2015 of the Praeventiefonds. We would like to thank Ms. Cindy Dole for her excellent editorial and technical skills in the preparation of this manuscript.

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