Fourth updated ESACP consensus report on diagnostic DNA image cytometry

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A task force of experts in the field of diagnostic DNA image cytometry, invited by the ESACP, and further scientists or physicians revealing experience in that diagnostic procedure (names are given in Addendum A), agreed upon the following 4th updated Consensus Report on Standardised Diagnostic DNA Image Cytometry during the 7th International Congress of that society in Caen, 2001. This report is based on the three preceding ones [6,14,17]. It deals with the following items:

- Critical review and update of the definitions given in the 1997 Consensus Update;
- Review and detailed description of basic terms, principles and algorithms for diagnostic interpretation;
- Recommendations concerning diagnostic or prognostic applications in specific fields of tumour pathology.

This update is not aimed to substitute the 1997 consensus, but to make necessary addenda and give more detailed descriptions of those items not unequivocally to interpret by potential users of the methodology.

1. Introduction

Cytogenetics have opened new sights in the understanding of tumour pathology during the last decade [1, 11,12,21,23]. It could be confirmed that chromosomal aneuploidy, characterised by numerical and/or structural chromosomal aberrations, is an early key event in tumorigenesis caused by genetic instability [4,13].

- The cytometric equivalent of chromosomal aneuploidy, DNA aneuploidy, serves as a marker of neoplasia by assessing large-scale genomic alterations resulting from genetic instability [24,28].
- DNA cytometry is furthermore able to monitor the effect of cytogenetic tumour progression on nuclear DNA content. Quantitation of DNA aneuploidy may therefore serve as a prognostic marker [2,7,8,11,29].
- Changes in DNA ploidy may indicate therapeutic effects [20].

As an example, recently an "International Consensus Conference on the Fight against Cervical Cancer" agreed that DNA image cytometry is indicated for the identification of prospectively malignant cells in squamous intraepithelial lesions and ASCUS, because chromosomal and DNA aneuploidy is consistent with high grade squamous intraepithelial lesions and cervical carcinoma. The finding of aneuploidy qualifies SIL therefore as high grade, needing further clinical management [15].

Having this in mind, the task force has critically reviewed the 1997 consensus reports as to check whether the definitions, algorithms and recommendations agreed upon, are still appropriate to be applied in tumour diagnostics and prognostication, and to allow exchange and comparison of results obtained by different laboratories.

2. Background and aims of DNA image cytometry

Quantitation of nuclear DNA content by cytometry has become practice for assistance in the diagnosis and grading of malignant tumours for some years. The DNA content cannot be measured directly by cytometry. After quantitative DNA-staining, the nuclear IOD

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(Integrated Optical Density) is the cytometric equivalent of its DNA content. Therefore the DNA content is expressed in a "c" scale in which 1c is half the mean nuclear DNA content of cells from a normal (non-pathological) diploid population in G0/G1 cell cycle phase.

For practical reasons as a term being accepted and used throughout the literature "DNA ploidy" will be further used. However, we want to point out that in practice the cytometric evaluation of nuclear DNA content is often improperly called "DNA ploidy" which is assumed to be the quantitative cytometric equivalent of "chromosomal ploidy". Both terms are not identical. Whereas "chromosomal ploidy" is theoretically detectable by cytogenetic methods in each single cell, its DNA content cannot be equated with a certain chromosomal outfit [25,26,30], but the term "DNA ploidy" is therefore the expression of the typical large-scale genomic status of a cell population. It can be the equivalent of that status also in single cells.

Indeed, the quantity of nuclear DNA may be influenced by the following mechanisms: replication, polyploidization, gain or deletion. Each affects the size or the number of chromatids. Viral infections may change the nuclear DNA content detectable by flow and image cytometry. Among others, the unspecific effects of cytostatic or radiation therapy, vitamin B12 deficiency, apoptosis, autolysis and necrosis on nuclear DNA content also play a role [3,9,24,27,31,32]. Furthermore, the DNA content of a cell is regularly changed throughout the cell cycle.

All these effects have to be taken into consideration when a diagnostic interpretation of DNA histograms is performed.

At present the basic aim of diagnostic DNA cytometry is to identify DNA stemlines outside the normal (euploid) regions as abnormal (or aneuploid) at a defined statistic level of significance. Furthermore DNA image cytometry should give information about

- Number of abnormal (sive aneuploid) DNA stemlines;
- Polyploidization of euploid or aneuploid DNA stemlines;
- Occurrence of rare cells with an abnormally high DNA content, most likely resulting from genomic alterations;
- Cell cycle fractions.

During the past few years a huge body of methodological experience has been gathered allowing ICM-DNA users to perform their DNA measurements at a high level of quality. Recommendations for the entire process of preparation and measurement have been given previously [17].

3. Principles of the method

Because DNA image cytometry results in nuclear IOD values in arbitrary units, equivalent but not identical with nuclear DNA content, the quantitation of nuclear DNA requires a rescaling of IOD values by comparison with those from cells with known DNA content, so-called reference cells. By means of reference cells the arbitrary unit scale (AU) will be transformed in a reference unit scale (2c, 4c, 8c, for example) [10,25]. In general, there are two types of reference cell systems: external and internal ones, respectively. Whereas the external reference cells are very easily to identify by the investigator, but often not to prepare in parallel with the clinical sample, the internal reference cells have the advantage of sharing all preparatory steps with the analysis cells in the clinical specimens.

The nuclear IOD values of reference cells own the same methodological limitations in terms of precision of the measurements as the appropriate IOD values of the analysis cells.

The mean ratio between the modal IOD values of the non-pathologic cells of the tissue under study and the reference cells used is called corrective factor. This corrective factor must be applied to DNA measurements from the clinical sample before any DNA histogram interpretation [25]. Due to the methodological variability, mentioned above, that ratio is not constant. The accuracy of each diagnostic DNA evaluation depends decisively on the standard error of the corrective factor used during the rescaling procedure [16].

Because most of the interpretations of DNA measurements are population-based, the results are usually displayed as DNA histograms. The bin size of such histograms should be adapted to the precision of the actual measurements, i.e., the lower the variability in the reference cell peak, the smaller the bin size of histogram classes could be.

The grammalogues "ICM-DNA" (image cytometric DNA) and "FCM-DNA" (flow cytometric DNA) are good descriptors used to designate the type of nuclear DNA measurement.

4. Basic performance standards

The usual precision of recent DNA image cytometric measurements should at least allow DNA stemlines to be identified as abnormal (or aneuploid), if they deviate more than 10% from the diploid (2c) or tetraploid region (4c), i.e., if they are outside $2c \pm 0.2c$ or $4c \pm 0.4c$.

To achieve this goal with an error probability $p < 0.05$ the test statistics [16] require a measurement performance described by:

- the cv of the ratios between modal IOD-values of reference cells and non-pathologic G0/1 cells in a series of measurements is <5%;
- the relative standard error (rSEM $= \mathrm{cv}/\sqrt{n}$) of reference cells in each sample is <1.5%.

Furthermore, a DNA-stemline should be identified as polyploid within the duplication position of a G0/1-phase-fraction $\pm 0.2c$ (at 4c), and $\pm 0.4c$ (at 8c), respectively, with an error probability $p < 0.05$ if

- the cv of the ratios between modal IOD-values of non-pathologic G0/1- and G2/M-phase-fractions in a series of measurements is <2.5%.

Every scientist and physician who applies DNA image cytometry is free to choose his appropriate methodological specification, if he only meets the performance standards agreed above.

The different aspects of the measuring process and of the interpretation should be regularly subjected to quality control measures in order to warrant a steadily high level of quality of the diagnostic procedure. Appropriate protocols for such a quality assurance guide have been described previously [14].

These protocols have also been implemented into the EUROQUANT quantitation server [18,19], adopted by the ESACP.

5. Definitions of basic terms of DNA image cytometry

DNA histogram

means a frequency distribution of IOD values obtained by cytometric measurements of cells stained stoichiometrically for their DNA and rescaled by IOD values from reference cells in "c" units.

DNA histogram peak

means a statistically significant local maximum in a DNA histogram. A recommended principle of finding and describing a peak by objective methods is given in the addendum.

Modal value of a DNA histogram peak

means the most frequent value in the peak, i.e. the mean value of that histogram class containing the highest number of nuclei. This is close or equal to the mean value of a fitted Gaussian curve according to the principle mentioned above.

DNA stemline

A stemline means a proliferating cell population with a unique chromosomal outfit.

A DNA-stemline is the G0/G1cell-phase fraction of a proliferating cell population (with a first peak and a second doubling one, or nuclei in the doubling region).

DNA euploidy

means that type of DNA distributions which cannot be differentiated from those of normal (resting, proliferating, or polyploidizing) cell populations.

DNA aneuploidy

means those types of DNA distributions which are different at a statistical significant level from those of normal (resting, proliferating, or polyploidizing) cell populations. DNA aneuploidy can either be seen as DNA stemline aneuploidy or can be indicated by "rare events" (see below).

DNA diploidy

Means that type of euploid DNA histograms which is the cytometric equivalent of a resting or proliferating cell population with a diploid chromosomal set.

Polyploidisation

Means the (repeated) doubling of a chromosomal set.

Euploid DNA polyploidisation means the occurrence of peaks in the duplication ($\times 2$, $\times 4$, $\times 8$, ...) regions of euploid stemlines. In human tissues usually the highest peak is at 2c.

Aneuploid polyploidisation means the occurrence of peaks in the duplication region of aneuploid stemlines.

Rare events in DNA histograms

are abnormal cells often called 5c or 9c exceeding events, having *a nuclear* DNA content higher than the duplicate or quadruplicate region of a normal G1/G0 phase population, i.e., not belonging to G2M phase. They likely represent non-proliferating abnormal cells with different chromosomal aneuploidies and abnormally high numbers of chromosomes.

DNA histogram typing

Histogram types are the result of a diagnostically and/or prognostically valid (i.e., statistically proven) classification of DNA distributions. It results in specific histogram types or classes (e.g., diploid, tetraploid, hypodiploid, aneuploid, etc., see also below).

DNA histogram grading in case of malignancy

The terminology for grading is only applicable to neoplasias, either proven by morphological investigations or in case of DNA aneuploidy. The prognostic interpretation of grading has principally to be tumour type specific. Grading can be performed as "histogram typing" using specific histogram types or by complex grading algorithms (see below).

The following *DNA histogram types* are helpful in the prognostication and monitoring of solid tumours:

- A peridiploid DNA histogram is supposed to with a stemline between 1.8c and 2.2c.
- A peritetraploid DNA histogram is supposed to exist in case of proven neoplasia or DNA aneuploidy and a stemline between 3.6c and 4.4c.

(The setting of the thresholds depends on the performance of the specific measurement, i.e., instrumentation and specimen preparation. The thresholds given above are therefore around 2c and 4c, respectively, $\pm 2 \cdot$ cv of the corrective factor (see below) of the system. They are around 2c and 4c, respectively, $\pm 10\%$ according the *minimal* performance standards, see above.)

- An x-ploid DNA histogram is supposed to exist in case of proven neoplasm and a stemline, alone or additional to a peridiploid/peritetraploid one, outside the thresholds mentioned above. "x" should be substituted by the DNA ploidy value of that stemline (e.g., triploid, hypertetraploid, or 2.6 ploid, etc.).
- A multiploid DNA histogram is supposed to exist in case of proven neoplasia and more than one stemline at positions outside the thresholds mentioned.

For each of those DNA histogram types the exact position of the stemline should be given. However, one has to take into consideration that the prognostic relevance of these classes may be different among the various tumour types.

6. Recommendations for clinical reporting on diagnostic DNA image cytometry

6.1. *Identification of neoplasia*

- Repetition of histological/cytological diagnosis;
- Indication for DNA-cytometry;
- Type of investigated material:
  - preparation,
  - type and number of reference cells,
  - type and number of analysis cells;
- Short description of the DNA-histogram (DNA histogram type);
- Interpretation of the DNA-histogram concerning the occurrence of DNA-aneuploidy and/or the histogram type;
- Summarised morphologic/cytometric diagnosis;
- Enclosures (DNA-histogram, relevant listing of indices of DNA-distribution, applied algorithms).

Remark. Changes of diagnoses in one-dimensional, unspecific nomenclatures referring only to the probability of presence of tumour cells are allowed (doubtful for malignancy → strong suspicion for malignancy → unequivocal malignancy).

Changes of morphological diagnoses in multidimensional, specific nomenclatures as the Bethesda system for reporting cervical smear diagnoses are not allowed as the given entities are only morphologically defined.

6.2. *Grading of tumour malignancy*

- Repetition of histologic/cytologic diagnosis;
- Indication for DNA-cytometry;
- Type of material:
  - preparation,
  - type and number of reference cells,
  - type and number of analysis cells;
- Short description of DNA-histogram;
- Prognostic interpretation of DNA-histogram;
- Summarised morphologic/cytometric diagnosis;
- Enclosures (DNA-histogram, relevant indices of DNA-distributions, applied algorithms).

Remark. Specific histologic or cytologic grades of malignancy (e.g., Bloom & Richardson for breast cancer, Gleason for prostatic cancer) should not be changed by discrepant DNA-results. Morphologic grades remain the same, despite deviating DNA-grades of malignancy.

Yet, the biological interpretation concerning the occurrence of malignant cells, or the malignant potential of tumour cells may be changed in a retrospective synopsis of morphologic and cytometric results.

Addendum A

The following scientists participated at the Consensus meeting during the 7th Congress on Analytical Cellular Pathology:
J.B.A. Baak, Stavanger, J. Belien, Amsterdam, A. Böcking, Düsseldorf, M.G.W. Bol, Alkmaar, A. Buhmeida, Turku, D. Chiu, Vancouver, Y. Collan, Turku, H.E. Danielsen, Oslo, J. Dufer, Reims, A. Elzagheid, Turku, J. Erenpreisa, Riga, N. Friedrichs, Düsseldorf, L. Giacomelli, Padova, F. Giroud, Grenoble, A. Gschwendtner, Innsbruck, T. Hanselaar, Nijmegen, G. Haroske, Dresden, M. Hubler, Basel, E. Janssen, Alkmaar, G. Jenkinson, Cambridge, P. Kiehl, Hannover, A.J. Kruse, Alkmaar, J. Lavrencak, Ljubljana, J. van Marsdyn, Amersfort, W. Meyer, Dresden, A. Reith, Oslo, F. Theissig, Dresden, J. van der Laak, Nijmegen, M. Vogelbruch, Hannover.

Addendum B

Algorithmic principles for understanding and following the definitions given above:

B.1. Finding a histogram peak [18,19]

(a) sort all measured IOD values in increasing order;
(b) count all measurement values inside a window of IOD ± 5%;
(c) move that window along the ranked measurement values, look for the window position with the highest number of values;
(d) test that maximal number Mnumb for significance:
    If (Mnumb · 1.5 > 6 · ($\log_{10}$(total number) − 1/$\log_{10}$(total number)) + 8)
    Then a peak was found;
(e) a peak was found:
    fit parameters of a Gaussian curve for
    - peak position
    - peak width
    - peak height

    to the measurement values inside the window, use the integral of the Gaussian curve (error function erf) for avoiding digitalisation of a histogram;
(f) subtract from the measurement values the values fitted to the peak;
(g) for searching the next peak go to (b)
    end if
    no further peaks found.

B.2. Coefficient of variation

The coefficient of variation is the the quotient of the standard deviation divided by the mean, given in percent:

$$cv = \frac{SD}{m} \cdot 100.$$

B.3. DNA-index

The modal value of a DNA stemline (or the mean of its fitted Gaussian curve) divided by the modal value of G0/1 peak of the reference cells.

B.4. DNA stemline ploidy [18,19]

The modal value of a DNA stemline (or the mean of its fitted Gaussian curve) in "c" units.

B.5. DNA stemline abnormality (aneuploidy) [16,18, 19]

An error probability p for belonging a peak modal value to the population of non-pathological peaks is given by:

$$p(t) = 2 \cdot \frac{1}{\sqrt{2\pi}} \cdot \int_t^\infty \mathrm{e}^{-x^2/2} \cdot \mathrm{d}x.$$

The test value t is given by:

$$t = \frac{|1 - M_A/(M_R \cdot cf)|}{\sqrt{cv_R^2/n_R + cv_A^2/n_A + cv_{cf}^2}},$$

where:

cf, corrective factor;
M_A, modal value of analysis cell peak;
M_R, modal value of reference cell peak;
cv_A, cv of analysis cell peak;
cv_R, cv of reference cell peak;
n_A, number of analysis cells in the peak;
n_R, number of analysis cells in the peak;

cv_{cf}, cv of the corrective factor.

If p is below a given threshold (e.g., 0.05, 0.01, 0.001) the classification of a peak as aneuploid (abnormal) is done with a false positive rate (FPR) of 5%, 1% or 0.1%, respectively.

B.6. Rare events [18,19]

The threshold above that the cells do not belong to a G2/M phase with an error probability of 0.15% is given by:

$$M_{A(d)} \cdot (1 + 3 \cdot cv_{A(d)}) \cdot cf_{poly} \cdot (1 + 2 \cdot cv_{cfpoly}),$$

where:

$M_{A(d)}$, modal value of the analysis cell peak classified as diploid(see above),
$cv_{A(d)}$, cv of the (diploid) analysis cell peak,
cf_{poly}, duplication factor of polyploidising populations, i.e., $M_{A(4c)}/M_{A(2c)}$,
cv_{cfpoly}, cv of the duplication factor.

B.7. Complex grading algorithms

The 2c Deviation Index (2cDI) [5]:

$$2cDI = \sum_{i=1}^{n} \frac{(c_i - 2c)^2}{n}$$

is the DNA content of a single nucleus, rescaled by the mean corrective factor of the tissue type under investigation.

The ploidy balance (PB) [22]:

$$PB = \frac{(n_{eu} - n_{an})}{N} \cdot 100,$$

n_{eu} is the number of all cells in euploid regions of the DNA histogram rescaled by the mean corrective factor of the tissue type under investigation (1.8c–2.2c; 3.6c–4.4c; 7.2c–8.8c); n_{an} is the number of all cells outside the euploid regions of rescaled DNA histogram; N is the total number of cells.

B.8. Finding optical disturbances (glare and diffraction effects) [16,18,19]

If the amount of the coefficient of correlation between nuclear area and IOD (DNA) in a single peak is greater than 0.40, then optical disturbances outside tolerable limits are assumed.

The coefficient r is calculated from all objects around the peak modal value (see 1.) $\pm 2 \cdot 5\%$.

B.9. Finding sampling inhomogeneities [18,19]

The proof of sampling inhomogeneities requires an unsorted sequence of IOD (DNA) values, originating from a stochastic sampling approach.

A sorting concerning time of acquisition (order of aquisition, resp.) allows conclusions for temporal or spatial inhomogeneity, a sorting concerning xy-coordinates leads to the same conclusions.

The evaluation of the entire sample gives hints for the distribution of reference cells throughout the sampling, the evaluation of peaks shows temporal and spatial inhomogeneities.

For each type of evaluation the sample (peak) is divided in 2 to 10 equal parts. In each part the distribution of IOD (DNA) values is tested non-parametrically (U-test) for being equal to the distributions of all other parts. After Bonferroni adjustage the error probability for being equally distributed is computed.

B.10. Rescaling of DNA values [18,19]

The rescaling is aimed at correcting systematic deviations from the theoretical 2c; 4c; 8c ratio of euploid cell populations. It allows the intercomparison of DNA values obtained under different technological conditions.

The actual ratios for the peak positions in a given technological condition should be determined by measurement of at least 36 non-pathological samples, comprising all peak regions of interest (usually 2c and 4c, sometimes 8c, too).

For each cell or peak a rescaling factor (corrective factor)

$$cf = \frac{2^i}{\overline{m}_i} + \frac{x - \overline{m}_i}{\overline{m}_{i+1} - \overline{m}_i} \cdot \left(\frac{2^{i+1}}{\overline{m}_{i+1}} - \frac{2^i}{\overline{m}_i} \right)$$

with $\overline{m}_i \leqslant x < \overline{m}_{i+1}$, where $\overline{m}_i$ is mean modal DNA value of the euploid peak$_i$ and $i = 1, 2, 3, \ldots$ is computed.

Measurement values around a peak$_i$ are all values greater than the geometrical mean between peak$_{i-1}$ and peak$_i$ as well as all values smaller than the geometrical mean between peak$_i$ and peak$_{i+1}$.

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