DNA Consensus in Image Cytometry

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- ***ESACP Consensus***

CONSENSUS REPORT OF THE ESACP TASK FORCE ON
STANDARDIZATION OF DIAGNOSTIC DNA IMAGE CYTOMETRY

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The following topics were agreed upon at the meeting of the above mentioned task force durinq the third conference of the ESACP, Grenoble, May 17th, 1994

This report refers to quantitative assessment of nuclear DNA by photometric methods on cytological material.

I. Biological background
Cytometric quantitation of nuclear DNA content is increasingly coming into practice for assistance in the diagnosis and grading of malignant tumors.

The quantity of nuclear DNA may be changed by the following mechanisms: replication, polyploidization, gain or deletion. Each affects the size or the number of chromatids. Furthermore gene amplification and viral infections may change nuclear DNA content in the limits of the resolution of flow- and image cytometry.

Among others, the unspecific effects of cytostatic therapy, radiation therapy, vitamin B12 deficiency, apoptosis, autolysis and necrosis on nuclear DNA content have to be taken into consideration when a diagnostic interpretation of DNA histograms is performed.

References: Böhm and Sandritter, 1975; Winkler et al., 1984; Tribukait et al., 1986; Shackney et al., 1990: Sandberg, 1990; Biesterfeld et al., 1994; Böcking et al.. 1994

II. Nomenclature to designate nuclear DNA measurements The grammalogues "ICM-DNA" (DNA measured by image cytometry) and "FCM-DNA" (DNA measured by flow cytometry) should be used to designate the type of nuclear DNA measurements.

III. Preparation of cytological specimens

1. Samples

Cell culture monolayers, imprints (touch preparations), smears from fine needle aspiration biopsies (FNAB), smears from exfoliated cells, cytocentrifuged preparations from body fluids, cell separation specimens (after mechanic and/or enzymatic dispersion) from FNABs, core- or other biopsies or from formaldehyde-fixed, paraffin embedded tissues may be used.

References:

Delgado et al., 1984; Hedley et al., 1985; Tutuarima et al., 1988; de Launoit et al., 1990; Heiden et al., 1991; Howat et al., 1992; Hutchinson et al., 1992; Geyer et al., 1993

2. Fixation

Fixation with formaldehyde is necessary before staining for Feulgen with pararosaniline or thionine. One possible procedure is to air dry the samples at room temperature for at least one hour. Then fix in 4% paraformaldehyde during 30 minutes and rinse in distilled water [4% paraformaldehyde = 4% (weight/vol) paraformaldehyde: 4 g polyoxymethylen in 100 Vol PBS (pH = 7,2) - heat to 70℃ - cool to room temperature - filter].

Prestained smears may also be used. Post fixation after uncovering is recommended according to the above mentioned procedure.

References:

Kotelnikow and Litinskaya, 1981; Baak, 1989; Giroud and Montmasson. 1989; de Launoit et al., 1990; Munck-Wikland et al., 1990; Aubele et al., 1994

3. Staining

Staining by the Feulgen reaction is recommended. Hydrolysis conditions for Feulgen have to take tissue type, fixation (time and concentration) and mode of preparation into account. Optimal staining conditions have to be worked out, based on hydrolysis curves (time vs IOD = integrated optical density). Hydrolysis has to be performed under controlled temperature and time conditions.
The following conditions are suitable for many routine applications: 5 M HCI, 25oC and 1 hour. Stop hydrolysis by rinsing in distilled water. Use Schiff reagent [pararosaniline (red) or thionine (blue)] during 1 hour. Check, that covered surfaces of slides are correctly exposed to reagents. Sulfite rinse to remove surplus dye from the cell nuclei and cytoplasm.

====== Absorpt. =Color Index =Color Index =Chem.Abstr.==Dye= Maximum ==
Name == == Number == == Number
Pararosaniline = 560 nm =basic red 9 =42500 =569-61-9
Thionine 590 nm =no C.I.name =52000 =78338-22-4

Commercial Schiffs reagent is often prepared from basic fuchsine which is a dye mixture of relatives of pararosaniline. The limit of shelf life of pararosaniline and thionine solutions is about one year and two weeks respectively.

Specificity control of the Feulgen reaction may be performed by staining an unhydrolysed specimen which must remain unstained.

Fluorochromes may also be used for DNA staining and subsequent measurements of fluorescence intensities.

References:
Feulgen and Rossenbeck, 1924; Graumann, 1953; van Duijn, 1956; Kjellstrand, 1977; Larson and Sanaia, 1980; Krug, 1980; Schulte, 1986; Chatelain et al., 1989; Schulte and Wittekind, 1990, a/b; Schulte, 1991: Mikel and Becker. 1991: Lyon et al. 1992

IV. Instrumentation requirements for densitometric measurements
Setting up the system:
1. Use different interference filters for blue (thionine, e.g. 590 +- 10 nm) or red (pararosaniline, e.g. 560 +-10 nm).
2. Köhler illumination.
3. Analogue and digital adjustment.

System quality assurance:
4. Check stability over time.
5. Check densitometric linearity
(e.g. using set of neutral glasses of defined transmissions).

6. Check for shading phenomena.
7. Check for glare phenomena.

References:
Echsner and Schreiber, 1962; Goldstein 1970; Bedi and Goldstein, 1976; Djuindam et al., 1980; Krug, 1980; Jarvis, 1986; Sanchez et al., 1990; Mikel and Becker, 1991; Kindermann and Hilgers, 1994; Cieco et al.. 1994. Reith and Danielsen, 1994

V. Densitometric measurements
1. Nuclei to be measured should be in focus. In automated systems, check on the image gallery after measurement.
2. No change of instrumentation adjustment during measurements (Köhler-illumination, analogue and digital adjustment).
3. Correct for shading by software procedures.
4. Correct for local background per nucleus by software procedures.
5. Correct for glare (= straylight) by software procedures.
6. Use visual control during and/or after measurements for artefact rejection and appropriate segmentation.
7. Check linearity of IOD values (2c, 4c, 8c), for example using cerebellum cells or rat liver hepatocytes.

VI. Reference cells
1. Reference cells are necessary for DNA scaling of densitometric measurements.
2. Use internal and/or external references. Lymphocytes, granulocytes, normal epithelial cells or stroma cells are usually analysed as internal standards. Rat liver 2c hepatocytes may be used as external standard.
3. Reference cells should be prepared and fixed identically as cells under analysis.

4. Reference cells should be stained in the same staining bath as the sample.
5. Reference cells should be analysed during the same run as the sample and under the same conditions.
6. The CV of the reference cell population should not exceed 6% (coefficient of variation = standard deviation ./. mean x 100).

References:
Tribukait et al., 1975; Munck-Wikland et al., 1990; Coen et al., 1992

VII. Scaling procedure
1. Use reference cells to transform the arbitrary unit scale (a.u.) in a reference unit scale (2c, 4c, 8c for example).
2. Make estimation of DNA measurement variations between the reference cells used and the diploid cells of the tissue under study and define a correction factor if necessary. Give standard deviation of correction factor.
3. Apply the corrective factor to DNA measurements from the sample before DNA histogram interpretation.

VIII. Performance standards
1. For measurements of one diploid cell at 30 different sites of the digitized field a CV of IOD values c 3% is an acceptable value under routine measurement conditions.
2. For measurements of a non dividing diploid cell population (n = 30, e.g. lymphocytes, granulocytes) at 30 different sites of the digitized field a CV of iOD values < 5% is an acceptable value.
3. Median IOD values of G0/G1-phase fractions of diploid cell types differing in nuclear size (at least 3 classes) should not differ more than 5% (e.g. granulocytes, myelocytes and erythroblasts from human bone marrow).

IX. Sampling validity and representativity
The number of suspicous or neoplastic cells to be measured depends on the type of histogram (number of peaks) and on the area of application.

X. Diagnostic and prognostic interpretation of DNA histograms
Simple and complex algorithms or classification strategies for histogram interpretation may be used for three purposes, depending on the material under investigation and the diagnostic or clinical questions:
1. diagnosis of neoplasia
2. prognostication of neoplasia
3. therapy planning of neoplasia.
The results of some of these algorithms depend on the number of histogram classes. Histogram classifications should not be based on subjective interpretations but be defined by algorithms:
The following algorithms and classifications may serve as examples.

DI DNA Index of Stemline (Hiddeman et al., 1984)
SLP Stemline ploidy (Böhm and Sandritter, 1975; Sandritter and Carl, 1966; Kropff et al., 1 991)
xER Rates of cells exceeding certain thresholds (x = e.g. 2,5c, 5c, 9c) (Böcking et al., 1984; Chatelain et al., 1989)
xEE Number of cells per specimen exceeding certain thresholds (x = e.g. 2,5c, 5c, 9c)

(Ploem-Zaaijer et al. 1979; Böcking, 1990, Böcking et al., 1993)
2cDI 2c Deviation Index (Böcking et al., 1984)
SSG Stemline shoulder fraction (Kropff et al., 1991)

Complex Algorithms:
DE Distribution Entropy (Stenkvist and Strande, 1990)
DNA-MG DNA Malignancy Grade (Böcking and Auffermann, 1986)
PB Ploidy Balance (Opfermann et al., 1987)
SLA Stemline Aneuploidy (Böcking et al., 1994)
AEd/t Aneuploid events diploid/tetraploid (Böcking et al.,1994)
DNA/RI DNA regression index (Böcking et al., 1985)

Classifications:
Albe et al. (1990): Colon **Tribukait** et al. (1983): Prostate **Böcking** et al. (1994): Kidney **Forsslund** and **Zetterberg** (1990): Prostate

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XIII. Addendum

The above ESACP Report is based on preceding activities during the Second and Third International Conferences on the Computerized Cytology and Histology Laboratory, Chicago, March 8-11, 1992 und March 6-9, 1994.

In 1992 a Task Force on Standardization of Quantitative Methods in Diagnostic Pathology has discussed under the guidance of Dorothy Rosenthal:

Group 1 on **Specimen Characteristics developed recommendations on specimen types and preparation, fixation methods, staining methods and controls**. These were limited to quantitative DNA analysis for clinical application using slide-based (microscopic) image analysis. Participants were: Robert L. Becker, Thomas Gahm, Klaus Kayser (coordinator), Ulrika v. Mikel, Sonya Naryshkin, Peter S. Oud, Noboru Tanaka and Rosemary E. Zuna.

Group 2 on **Instrumentation contributed data acquisition standards (optical system requirements and photometric standards), standards for data computed from images, software and editorial standards,** all relevant for DNA image cytometry. Participants were: Peter H. Bartels, Daniel F. Cowan (coordinator), Harvey E. Dytch, Lawrence L. Hause, Manuel Hilgarth, Robert S. Ledley, Martin Oberholzer, Johan S. Ploem, George L. Wied and David J. Zahniser.

Group 3 on **Biology-Gynecology dealt with the possible application of diagnostic DNA measurements in neoplastic lesions of the uterine cervix, ovary and endometrium**. Participants were: Mariuce Bibbo, Ton A.G.J.M. Hanselaar, Martha L. Hutchinson (coordinator), Toshihiko Izutsu, Leopold Koss, Iwao Nishiya, Ralph M. Richard and G. Peter Vooijs.

Group 4 on **Nongynecologic Tumors elaborated recommendations concerning the clinical usefulness of DNA measuarements in diagnostic cyto- and histopathology in nongynecologic tumors**. Participants were: Alfred B_cking (coordinator), Kenney W. Gilchrist, Gianmario Mariuzzi, Ulrich Schenck and Alain Verhest.

Final agreements could not be achieved on the drafts which were prepared during the sessions of the respective Task Force groups.

In March 1994 at the Third Conference on Computerised Cytology and Histology Laboratory in Chicago a Task Force on DNA Standardisation was coordinated by Albrecht Reith. Topics addressed were: Biological background, preparation (samples,

fixation, staining), instrumentation requirements (including performance standards and measurements), calibration and diagnostic interpretation.

Members of the editorial committee for the final draft were: Daniel F. Beals, Alfred Bocking, Harvard E. Danielsen, Robert C. Leif, Ulrich Schenck and Albrecht Reith (coordinator) .

Much agreement on the outlines of the concept of DNA standardisation was achieved and founded the structure and basic components of the above ESACP Consensus Report conceived in May 1994 in Grenoble at the 3rd ESACP conference.

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