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Original Paper

Competence on Demand in DNA Image Cytometry*

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Summary

Quantitation methods in clinical pathology have to be normalized and standardized both from the instrumental and the methodological point of view to guarantee a defined level of precision and accuracy independent of the site where they are applied. The comparability of results obtained in different laboratories is the basis for the application of standardized diagnostic classification systems and therapeutic schemes. Remote quantitation based on standardized evaluation tools could be a way to reach the goals mentioned above. Diagnostic DNA image cytometry, increasingly used as a routine method in clinical pathology, will serve as an example for demonstrating the feasibility and usefulness of a concept of remote quantitation. We report a system for a remote DNA ploidy analysis, based on client server technology, and accessible via Internet or ISDN connections (Quantitation Server EUROQUANT). This system (i) allows the cytometric measurement of the DNA content of cells for diagnostic purposes, (ii) provides the user with comprehensive quality control of such measurements, (iii) helps in trouble-shooting, and (iv) gives assistance in diagnostic interpretation. The system uses the principles of telepathology and Internet technology. To date, more than 40 laboratories from Europe, USA, and Asia have successfully performed analyses on about 3,000 ploidy data sets

Key words: DNA ploidy – Tumor diagnostics – Quality control – Standardization – Telepathology

Introduction

DNA image cytometry, which is increasingly applied by pathologists, is a useful diagnostic tool. It helps in grading malignant tumors and in identifying malignancy in borderline lesions and dysplasias.

Methodologically, DNA ploidy analysis can be subdivided into the three following basic steps: (1) preparation of specimens, (2) cytometric measurements, and (3) diagnostic interpretation of the cytometric data. Possible errors in all of these steps of DNA ploidy analysis that are neither identified nor adequately corrected by the user can result in misdiagnosis with disastrous clinical consequences. Clinical pathologists applying DNA cytometry are often not able to check all key elements of their laboratory practice and the performance of their image analysis workstation. For optimizing the quality of DNA analysis, standardized methodological tests (for steps 1 and 2) and assistance in the diagnostic interpretation of the cytometric results (for step 3) are imperative.

On the one hand, consensus guidelines on the standardization of diagnostic DNA image cytometry have already been drawn up *[5, 9]*. On the other hand, the diagnostic interpretation of DNA measurements may be very complex *[2, 6, 16]* and requires both biological and methodological knowledge at a high level of competence. Finally, standardized, quality-controlled DNA ploidy data are essential for multicentric studies in cancer research.

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To date, the different devices for performing cytometry available on the market are not compatible with each other, nor can their results be directly compared. Therefore, standardized quality control procedures are still problematic. Besides a few device-specific quality control programs [1, 15, 21] (http://www.mcs.net/~bacuslab/home.html) and user clubs [22], DNA cytometry measurements have not yet been checked for their consistency with recommendations for good laboratory practice. Remote quantitation based on standardized evaluation tools could pave the way for solving these problems.

We developed a client-server system for remote DNA ploidy analysis [8, 10] that provides the following benefits: (1) DNA measurements can be compared and normalized, independent of machinery used for data acquisition; (2) this occurs objectively, without human interaction at the most recent know-how; (3) diagnostic conclusions can be drawn on a statistical basis; and (4) the quality control can steadily be used in routine diagnostic DNA image cytometry.

The key elements of such a client-server system are:

1) a database for the documentation of the cases, together with their DNA raw and result data,
2) a private mailbox for each user, collecting the incoming and outgoing data,
3) a computer with algorithms for data handling, computation of ploidy data, and quality control,
4) a communication network.

Server and Clients

The server "EUROQUANT" was designed to serve both as consultation system and as cytometry workstation that can be used remotely via Internet technology. Its main functional components are:

- analysis of the measurement performance of DNA ploidy analysis in all of its steps, leading to methodological recommendations,
- confirmation or revision of the diagnostic interpretation of the data obtained by the user,
- rescaling of DNA data for data exchange with multicenter databases.

The **server** (http://euroquant.med.tu-dresden.de) is based on a PC-system with INTEL Pentium® 200 CPU, 128 MB RAM and 4 GB HD capacity, equipped with WINDOWS NT®4.0.

A system of functional modules has been constructed for:

- data exchange via Internet or ISDN,
- multimedia mail boxes for images, measurement and resulting data,
- databases for processing data and results,
- DNA ploidy analysis,
- performance control,
- teaching and training.

Pathologists operating a DNA image cytometry device, a telepathology workstation, or a simple PC with imaging facilities can use the quantitation server free of charge. Any Internet browser allows the **clients** of EUROQUANT to gain access to the peripheral devices or workstations. The clients send microscopic images and/or DNA measurement data to the server, which executes process commands from the clients, and provides numerical results and graphic displays for visualization and downloading.

Remote Data Analysis

DNA image cytometry measurements are evaluated automatically without human interaction at the server's site. Two different options are offered to have diagnostic specimens analyzed:

1) By sending microscopic images grabbed from specifically stained specimens to the server for measurement and analysis;
2) By sending measurement data (integrated optical density, nuclear area, and coding of each measured nucleus) obtained by any DNA cytometry device to the server for data analysis and quality control in a standardized procedure.

Both options are illustrated in Fig. 1. No specific hard- or software is needed by the user, only a telepathology / cytometry workstation equipped with an Internet browser is necessary. For more privacy, the user can also have ISDN access with the same Internet technology or Internet SSL encryption procedures.

The principal sequence of the steps in using the server never changes: (1) transfer of images or data to the server, (2) import into the server's database, (3) DNA ploidy analysis, check of performance standards as well as quality control, and (4) download of the results.

After the images have been transferred to the server's mailboxes, the user can operate the server by his client

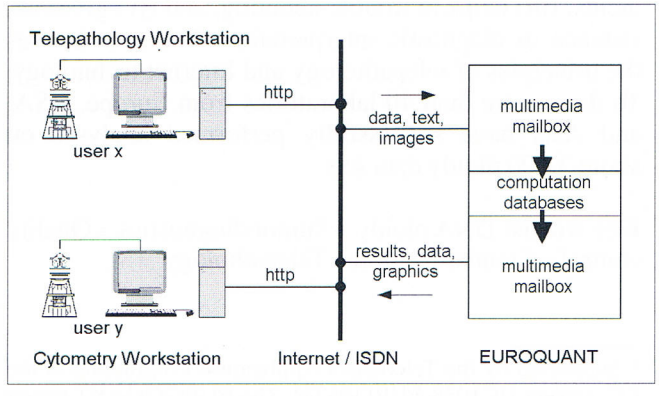

Fig. 1. Access to the quantitation server is possible by different types of users via the Internet.

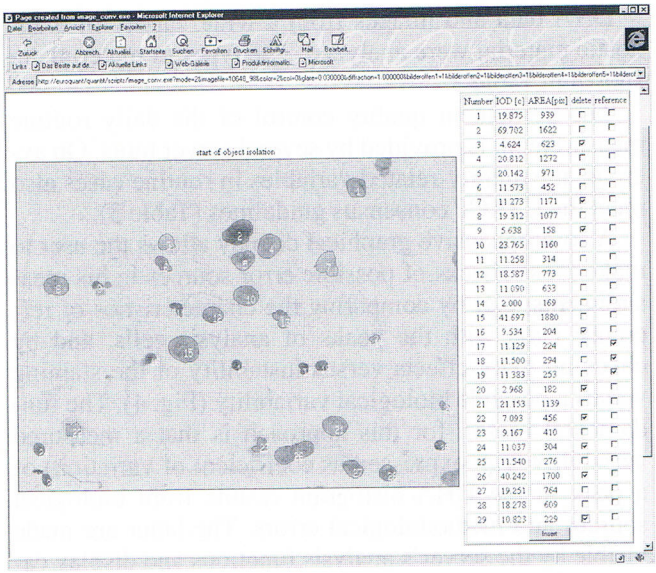

Fig. 2. A webpage with segmented nuclear images of a Feulgen-stained breast cancer imprint. In the table measurement values and boxes for deleting cells are displayed. The objects 17, 18, and 19 are referred to as "reference cells", whereas the objects 3, 7, 9, 16, 20, 22, 24, 26, and 29 should be excluded from measurement.

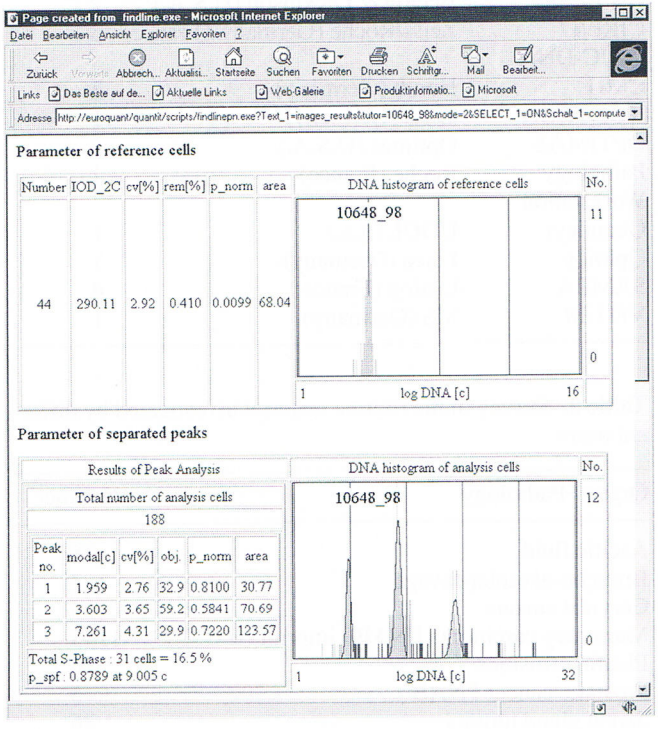

Fig. 3. A webpage with graphs and numerical data of a DNA ploidy analysis on a single specimen (the same as in Fig. 2), after quantification of a series of ten images.

computer for remote quantitation. The images are automatically segmented, and the segmentation masks are displayed to the user together with a table of options for selecting reference cells, or for deleting objects from analysis (Fig. 2). Any serious error in the gain and offset control of the imaging device at the client's workstation is indicated in the processed image at the server and announced to the user.

Analysis of the data gathered from images at the server or transferred from the client cytometry workstation is started by an import of the key data (nuclear area, integrated optical density) from each cell into the server's databases. The functions of the server yield a comprehensive selection of primary parameters (numerical data and graphs), backed up by a wide range of quality assurance data, according to generally accepted guidelines for good measurement practice. Fig. 3 shows how the results are presented. All values exceeding the guideline thresholds (recommended by the ESACP [5, 9]) are flagged; help is offered for finding the reason for these deviations, e.g. an undercorrected glare effect in the microscope.

The results are stored in a multimedia mailbox of the server, from where they can be downloaded by the user at any time.

Quality Control Tools and Diagnostic Advice

A user may have quality control tools at different levels of the cytometric and diagnostic processes. All of them share the common evaluation of primary DNA measurements by the server. (1) Technological settings of the client's cytometry workstation can be tested by the remote image quantitation tools. (2) The user can check the stability of the preparation, fixation, staining, and further technological aspects of the cytometry process by means of calibration material and check sample specimens, as recommended by the European Society of Analytical Cellular Pathology [5]. (3) The quality of the measurement of each single specimen is checked steadily during the normal analysis (see below). (4) For scaling, the DNA axis quality control is provided by several Levy-Jennings charts, built up from results of the user's own measurements held at the databases of the server. (5) A series of images from diagnostic specimens is held in the multimedia mailboxes for testing the diagnostic vigilance of the users.

Having analyzed a certain amount of specimens with non-pathological conditions among them, the user can have classifiers calculated for the diagnostic evaluation of a given type of specimen. These classifiers should be used for the discrimination of normal versus abnormal specimens (DNA-euploid versus DNA-aneuploid), as well as for a rescaling of the primary measurement data into units of "c" (DNA content). By these procedures,

DNA data can be compared with those from other users obtained by quite different cytometry devices, because such data are highly standardized.

The server works completely independently of any commercially offered cytometry device. Its functionality is based on the latest scientific knowledge and consensus agreement in the field. Therefore, the server is an objective tool for an international methodological standard.

Data Privacy and Response Time

An invaluable precondition for a practical application of such an internationally available quantitation server is the absolute preservation of the user's privacy. Research-oriented data analysis in the server databases is accessible for users only. Their privacy is protected by an authorization process with personal passwords, and by a firewall in front of the server, preventing unwanted access to the user's mailboxes. A still higher degree of data protection, also during the data transfer, can be obtained by using ISDN access instead of the Internet. Although the use of ISDN increases the communication costs, it does not have any influence on the hard- and software configuration.

Use of the Service for Routine Purposes

To date, more than 40 laboratories from Europe, USA, and Asia, working with 13 different cytometry systems, have been registered and authorized at the quantitation server, and have been successful in performing analyses on about 3000 ploidy data sets. For five of them, the server has been incorporated in the quality control of daily routine in DNA image cytometry. The server is online free-of-charge 24 hours a day. In Table 1 the variety of image cytometry workstations operated by registered EUROQUANT users is given.

On average, a dialogue between the user and the server for transferring and importing measurement data of one specimen, as well as for calculation of a DNA histogram, takes less than two minutes. The calculation of histogram data and their display require less than 10 seconds. Image transfers require more time, and are dependent on the type of transfer medium and the image size.

Several rounds of Quality Control Programs, run according to the ESACP recommendations *[5]* by some of the users, demonstrated a sufficient level of precision and accuracy in the measurements performed by most users. With users failing to meet the guideline threshold, a second run showed that a feedback occurs while spotting systematic errors, leading to successfully passing the repeated tests.

DNA data and images from a wide variety of diagnostic material were analyzed. The types of material are shown in Table 2.

The permanent quality control of the daily routine measurement is provided by several server tools. On average, the quality-related variables in routine cases also meet the ESACP consensus guidelines (Table 3).

A comprehensive graphical display allows the user to make cross checks of possible error sources in his measurements, e.g. by comparing the characteristics of reference cells with the peaks of analysis cells, and by checking glare effects versus instability of the staining versus a possible biological variability (Fig. 4). The fundamental reason for this approach is that a measured variability (e.g. expressed as coefficient of variation) of a peak in the DNA histogram results from biological sources and methodological errors. The latter are made visible by the server's analysis functions and display ca-

Table 1. Image cytometry systems operated by EUROQUANT users

System	Delivered by	Number of users
AxioHOME	Zeiss/Alcatel (Germany / France)	3
ACAS	Ahrens (Germany)	1
CAS 160	Becton & Dickinson (U.S.A.)	3
CAS 200	Becton & Dickinson (U.S.A.)	2
CIRES / QUIC-DNA	Zeiss/Roche (Germany / France)	3
CM-1	Hundt (Germany)	3
KONTRON	Zeiss (Germany)	1
OPTIMAS	Optimas (U.S.A.)	5
Pathology Workstation	Roche (France)	1
Quanticyt	UDDL (n.a.)	1
Qploidy	Leica (Germany)	1
SAMBA	Unilog (France)	4
VITUM	SIS (Germany)	1

Table 2. Survey of the routine material investigated by different users

Organ / Pathology	Number
Ascitic fluid	25
Broncho-alveolar-lavage	2
Cervical smears	223
Mammary carcinoma FNAB / imprint	1143
Pleural effusions	76
Prostatic cancer FNAB	5
Sweat gland tumors	86
Thyroid nodules FNAB	8
Urinary bladder washings	96
Oral cavity tumors	191

Table 3. QC variables (median values) of individual measurements in 17 users.
cv – coefficient of variation [%], rem – relative error of the mean [%], corr coeff – correlation coefficient *r*; p homogeneity – error probability *p* that a sequence of measurements is randomly distributed (see Fig. 4); * – no recommendation

Variable	Minimum	Mean	Maximum
Number of reference cells	**10**	24	101
cv of reference cells	1.31	3.69	**6.66**
rem of reference cells	0.19	0.73	**2.21**
corr coeff area vs IOD	0.01	0.28	**0.77**
p homogeneity	**0.004**	0.058	0.45
Number of analysis cells*	125	257	312
cv of analysis cells*	1.97	3.94	9.49

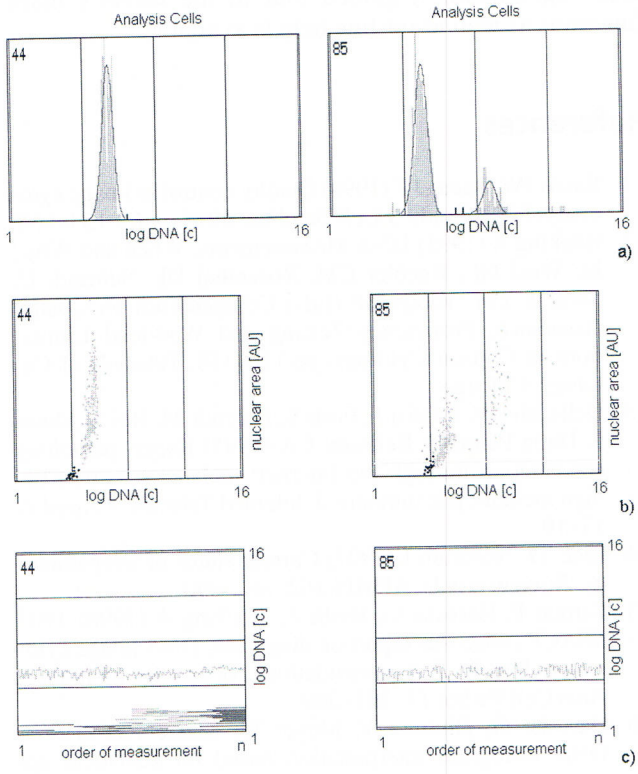

Fig. 4. Part of the result display dealing with internal quality control issues.
a) DNA histograms of two samples from breast cancer imprints.
b) Scattergrams of nuclear area vs. DNA content in these two samples. The deviation of the ellipsoid from the vertical axis, expressed as correlation coefficient *r*, indicates glare and / or diffraction effects beyond the ESACP guidelines in both specimens ($r = 0.56$ in 44; $r = 0.67$ in 85). These effects also lead to a shift in the modal peak value of analysis cells to the right (compared with the smaller reference cells, printed as black dots).
c) Sequential displays of the DNA content of cells within a histogram peak (same measurements as in a), showing a statistically significant departure from homogeneity in sample 44 ($p = 0.000$ in 44; $p = 0.26$ in 85)

pabilities. Fig. 4 reveals influences in two distinct measurements performed in breast cancer imprints, showing considerably high peak cv. In both measurements, glare / diffraction effects, beyond the ESACP guidelines, are responsible for the high peak cv. In sample 44, an inhomogeneous sampling has an additional share in the cv of the peak. This is indicated by the statistically significant departure from homogeneity, shown by horizontal bars beneath the graph of the sequential DNA-values (Fig. 4c). The DNA values on the right are systematically higher than those on the left.

Discussion

In recent clinical pathology, additional methods for diagnostics and prediction of prognosis are increasingly used. Due to the high complexity of such methods, the pathologist cannot be equally familiar with all of them. Not only indication may be critical, but also the methodological skills and the diagnostic interpretation often require advice from specialists from centers of competence. However, most of the pathologists involved in routine diagnostics will ask sporadically for advice. A close collaboration between these pathologists and special centers is therefore rather exceptional. With the upcoming telepathology, the current paradigms could change.

Among the application fields of telepathology, remote quantitation is a comparatively recent one *[7, 12]*. Because telepathology is currently almost exclusively based on the exchange of digitized images *[4, 17]*, the parallel use of cytometric or similar techniques, together with image transfer, seems to be a logical consequence for solving special problems in clinical pathology *[18, 19]*. However, the quantitation of such digitized images is faced with several difficulties, mostly with the lack of interoperability and methodological comparability of a rather broad spectrum of existing devices for cytometry and morphometry. Therefore, a client-server concept has been designed to overcome such obstacles *[8]*. This concept is based on the technological state of the art for existing cytometry workstations in university institutes, hospital departments, and private institutes of pathology throughout the world, as well as on the need of clinical pathologists to get high level assistance in the application of special methods for diagnosis, and on the existing achievements made in the standardization of such methods. The quantitation server does not only allow a remote quantitation of DNA data, but it also facilitates those analyses performed without any human interaction, i.e. in an objective way.

By means of such analyses, the pathologist is provided with quality-controlled proposals for classifying or typing a certain DNA-histogram. The final diagnostic decision, however, is exclusively made by the pathologist.

A further important aspect is the fact that such a non-attended service can be used at any time, compared with traditional scenarios of remote consultation with an expert available in time [3, 14].

Furthermore, the server is able to act as an interface for contributors of data to centralized tumor marker data bases, and for an editorial board evaluating the entries made to this marker data base.

As such a "neutral" platform a quantitation server may allow a very practical application of the concepts in quality control, quality assurance, and good laboratory practice in pathology [13], particularly in diagnostic DNA cytometry [7, 20, 24]. To our knowledge, a comparable service for remote analysis of cytometric performance, quality assurance, and diagnostic assistance in DNA cytometry has not been established so far. Very recently, an ATCDI server (http://pceuropath. imag.fr/), based on a similar concept like EUROQUANT, has gone into operation for test runs.

The acceptance of the quantitation server EUROQUANT and its performance show a quite new quality in the applicability of diagnostic DNA image cytometry. The functions of the server still have to be adopted to additional demands of the users and to scientific recommendations of international societies. Detailed written instructions for the use of EUROQUANT can be downloaded as a manual directly from the server. With all detailed improvements of the functionality, this manual is updated regularly.

In ongoing studies with a broader spectrum of users, some variables are now being analyzed for their correlation with diagnostically relevant characteristics of the DNA histograms. This may also include an updated proposal for consensus guidelines.

The most important benefit of a remote quantitation system is obvious: having calculated interactively diagnostic classifiers, the user is able to rescale DNA measurement. They lead to DNA ploidy data that can be directly compared with data from other sources. These DNA data can be compiled into large multicenter studies, because the DNA variables are absolutely standardized, quality controlled, and independent of any special cytometry system.

The EUROQUANT facility has demonstrated that a remote quantitation server is a valuable addition to the range of telepathology tools [8, 11, 23] available on demand to the clinical pathologist. It is likely that this concept can be extended to other fields involving quantitation, such as immunocytochemistry and molecular medicine.

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Appendix

The server EUROQUANT can be accessed via the Internet URL
http://euroquant.med.tu-dresden.de
or via ISDN by dialing +49-351-458 8443 . For this access, the user should have remote data transmission facilities with TCP/IP PPP synchronous.

From the WebPages a manual can be downloaded after registration. A guided tour to the server's main functions as well as on-line help is available.

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