Slit Scan Flow Cytometry of Isolated Chromosomes Following Fluorescence Hybridization: an Approach of Online Screening for Specific Chromosomes and Chromosome Translocations

Michael Hausmann[a], Gertrud Dudin[a], Jacob A. Aten[b], Rainer Heilig[a], Eduardo Diaz[a], and Christoph Cremer[a]

[a] Institut für Angewandte Physik, Universität Heidelberg, Albert-Überle-Straße 3–5, D-6900 Heidelberg, Bundesrepublik Deutschland
[b] Laboratory of Radiobiology, University of Amsterdam, AMC, Meibergdreef 9, NL-1105 AZ Amsterdam, The Netherlands

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The recently developed methods of non radioactive *in situ* hybridization of chromosomes offer new aspects for chromosome analysis. Fluorescent labelling of hybridized chromosomes or chromosomal subregions allows to facilitate considerably the detection of specific chromosomal abnormalities. For many biomedical applications (*e.g.* biological dosimetry in the low dose range), a fast scoring for aberrations (*e.g.* dicentrics or translocations) in required.

Here, we present an approach depending on fluorescence *in situ* hybridization of isolated suspension chromosomes that indicates the feasibility of a rapid screening for specific chromosomes or translocations by slit scan flow cytometry.

Chromosomes of a Chinese hamster × human hybrid cell line were hybridized in suspension with biotinylated human genomic DNA. This DNA was decorated with FITC by a double antibody system against biotin. For flow cytometry the chromosomes were stabilized with ethanol and counterstained with DAPI or propidium iodide (PI). An experimental data set of several hundred double profiles was obtained by two parameter slit scan flow cytometry and evaluated automatically. The evaluation algorithm developed allowed a classification of chromosomes according to the number of centromeres and their chromosomal positions in less than 1 msec per individual profile. Approximately 20% of the measured DAPI profiles showed a bimodal distribution with a significant centromeric dip indicating a "normal" chromosomal morphology and a correct alignment in the flow system. In many cases, profiles of a "normal" bimodal fluorescence distribution of the DNA stain (DAPI, PI) were correlated with a "normal" FITC profile. Due to their centromeric indices these profiles agreed well to the expected human chromosomes of the cell line. In some cases of "normal" DAPI (PI) profiles, "aberrant" FITC profiles were observed. These were interpreted as interspecies translocation chromosomes. For all results, there was a good agreement between flow cytometry and microscopic observations (digital image analysis).

Introduction

Flow cytometry ("flow karyotyping") has become an important tool for fast quantitative classification and sorting of isolated chromosomes in suspension due to the fluorescence intensity of DNA specific dyes [1–6]. By means of slit scan flow cytometry [7] the number of centromeres or the centromeric index of each chromosome can be obtained as an additional classification parameter [1, 2, 7–12]. The fluorescence profile (relative fluorescence intensity *vs.* time of flight the chromosome needs to traverse the detection volume) of a chromosome stained with a DNA specific fluorochrome (for instance DAPI, PI) shows a typical dip in the centromeric region. This allows, for example, the rapid detection of dicentrics following X-ray irradiation [10].

Non radioactive *in situ* hybridization [13–15] has already found interesting applications in the classification of chromosomes on slides. Due to the specificity of the given DNA probe, chromosomal subregions can be labelled in interphase chromatin or metaphase chromosomes [15–23]. The recently developed technique of chromosomal *in situ* suppression (CISS) hybridization [24–27] or "chromosome painting" [28] allows the delineation of entire chromosomes using chemically mod-

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ified unique DNA sequences from a chromosome specific DNA library [6]. This method offers new approaches for aberration screening for example in cases of radiation exposure [29, 30].

In situ hybridization to metaphase chromosomes in suspension [31, 32] has been successfully applied for instance to magnetic separation of chromosomes [33, 34]. In these studies, chromosomes from Chinese hamster × human hybrid cell lines were used. So far, however, a detailed analysis of isolated chromosomes following fluorescence hybridization in suspension has been performed only microscopically. Preliminary results using conventional flow cytometry indicated that under certain experimental conditions the morphology of the hybridized chromosomes can be preserved for flow karyotyping [3]. Other preliminary accounts showing that in principle it is possible to study hybridization of chromosomes in suspension by slit scan flow cytometry have been reported earlier by us [2, 35]. Here, a more detailed description as well as an automated evaluation of the analysis of such chromosomes is presented using two parameter slit scan flow cytometry. For comparison, digital image analysis of randomly selected microscope images of human and Chinese hamster chromosomes was performed.

Materials and Methods

Isolation of metaphase chromosomes and in situ hybridization in suspension

Chromosomes of the Chinese hamster × human hybrid cell line A_1wbf_2 (containing mainly the four human chromosomes X, 11, 17, 12/21 and several interspecies translocations) were isolated with a hexanediol method [36, 37] and hybridized with biotinylated human genomic DNA in suspension as described elsewhere [31].

Briefly, the cells were grown on Ham's F10 medium supplemented with 10% fetal calf serum, 1% hypoxanthine/thymidine solution (50 × HT concentrate) and 1% penicillin/streptomycin (10,000 U/10,000 µg/ml) solution. After 8 h of a demecolcine (Colcemid) block (0.25 µg/ml medium) followed by another cell cycle in the absence of Colcemid, the synchronized mitoses were harvested by shake off. After centrifugation (350 × *g*, 15 min), the cell pellet was suspended in a hypotonic solution (10 mM Tris, 5 mM $MgCl_2$, 10 mM NaCl, pH 7.5) for 15 min at room temperature. The hypotonic solution was removed by centrifugation, and the cells were resuspended in 1 ml hexanediol isolation buffer (pH 3.2, 25 mM Tris, 750 mM hexanediol, 5 mM $MgCl_2$, 5 mM $CaCl_2$). To suspend the chromosomes, the mitotic cells were sonicated. The isolation buffer was removed by centrifugation (350 g, 15 min), the chromosome pellet was washed in 1 ml 2 × SSC (1 × SSC: 0.15 M NaCl/0.015 M sodium citrate) and transferred to a Falcon tube no. 2054. After centrifugation the chromosome pellet was resuspended in 0.5 ml hybridization mixture (40% deionizied formamide, 4 × SSC, 2 × Denhardt's solution, 10% dextran sulfate, 1 µg human genomic DNA biotinylated by nick translation). The chromosomes were denatured by 73 °C (6 min) and incubated at 38 °C (in some experiments at 42 °C) for hybridization (*ca.* 15 h). After centrifugation (350 × *g*, 10 min) the hybridized chromosomes were washed in 1 ml prewarmed 2 × SSC (30 min, 30 °C or 42 °C) centrifuged, washed in 1 ml 0.1 × SSC, again centrifuged and resuspended in 2 × SSC.

Fluorescence labelling and preparation for slit scanning

The hybridized chromosomes were centrifuged and resuspended in 1 ml IB + M buffer (50 mM KCl, 5 mM Hepes, pH 8.0, 10 mM $MgSO_4$) [38]. For FITC labelling of the human chromosomal material, a double antibody fluorescence detection system (ENZO, Neckargemünd, F.R.G.) was used and the chromosomes were stained as described [31]. The hybridized and FITC labelled chromosomes were stored at 4 °C. About 30 h later the chromosomes were monitored in the light microscope after counterstaining with DAPI (5 µM) or PI (75 µM).

Aliquots of 200 µl of the chromosome suspension were used for flow cytometric studies. To obtain a suitable concentration for slit scan flow cytometry, 200 µl IB + M buffer were added. The chromosomal morphology was stabilized by the addition of 120 µl absolute ethanol (30% of the suspension volume) [39]. To avoid clumping, this had to be done dropwise under continuous pipetting. The suspension was then stained with PI (75 µM) or DAPI (5 µM). 10 µl fluorescence anti-

fading buffer (1 mg *p*-phenylene-diamine in 1 ml glycerine buffer, pH 8) was added.

Instrumentation for digital image analysis

For the analysis of the microscopic image of chromosomes following fluorescence hybridization in suspension, microphotographs (diapositives) were taken through a 515 nm long pass filter and digitized using a drum scanning densitometer (Scandig 2605, Joyce Loeble). For each chromosome, the sum of the gray level values of any line perpendicular to the chromosome axis was calculated. The evaluation of these integrated density profiles was carried out on a VAX 11/780 computer. With a centromeric dip visible in these profiles, the centromeric indices (CI) were calculated in two different ways:

1.) Length of the long arm/entire chromosome length;
2.) sum of gray values of the long arm/sum of gray values of the entire chromosome.

Between both ways of evaluation a good linear correlation was obtained (see also [35]).

Instrumentation for slit scan flow cytometry

Two parameter slit scan flow cytometry was performed on the Amsterdam slit scan flow analyzer based on an Ortho Cytofluorograph 30 H as described by [11, 12]. The optical resolution of the slit scan system was 2.7–3.0 µm (full width at 1/e points of the laser beam profile) using a 200 µm flow cell (Ortho Systems 30/50). Flow speed was about 2–3 m/sec. For DAPI excitation an argon ion laser was tuned to 410 mW and UV multiline (337–364 nm). PI and FITC excitation were performed with an argon ion laser at 488 nm with 500 mW. The time delay between the excitation by the first and by the second laser beam was 70 µsec. If the chromosomes were counterstained with PI, only the visible excitation laser beam was used. In this case, the PI fluorescence was collected on channel 1, while the FITC fluorescence was collected on channel 2 *via* a 525 nm band-pass filter of 10 nm half width. "Cross talk" between the channels was negligible.

Data analysis

The profile data were transferred to an IBM compatible personal computer with an 80386 microprocessor, cache memory, and a 25 MHz clock. For evaluation, an assembler program was written, which allows online profile classification according to the centromeric index or the number of centromeres in less than 1 msec per individual profile applying a threshold procedure to several characteristics of the measured profile and the second derivative analogue of the profile. The CI was calculated by:

Fluorescence intensity of the long chromosome arm/total fluorescence intensity of the chromosome.

For two parameter slit scan flow cytometry, the profile of the second fluorescence parameter can be evaluated accordingly. Hybridized chromosomes can be distinguished by a threshold procedure using the differential profile of the two normalized fluorescence profiles.

The algorithm was developed from the recently published program for fully automated slit scan data analysis using a parallel computer [40, 41]. Further details will be published elsewhere (R. Heilig *et al.*, manuscript in preparation).

In some earlier experiments a detailed computer analysis of the data was not practicable for technical reasons. In these cases the profiles were inspected manually and classified as chromosome profiles if the following criterion was fulfilled for DAPI/PI counterstaining [2]:

$$(I_{\max I}/I_{\min})/(I_{\max I}/I_{\max II}) > 1.1.$$

$I_{\max I,II}$: 1st and 2nd maximum of the fluorescence intensity;

$I_{\min}$: fluorescence intensity in the centromeric region.

Results and Discussion

Microscopy and digital image analysis

For microscopy the FITC labelled hybridized chromosomes were counterstained with PI or DAPI. This resulted in green-yellow or green fluorescent human chromosomal material (observation through a 515 nm long pass filter). In contrast, the Chinese hamster material revealed red fluorescent (PI counterstaining; observation through a 515 nm long pass filter) or blue fluorescent (DAPI counterstaining, observation through a 430 nm long pass filter). The morphology of the chromosomes dropped on slides after the hybridization procedure in suspension was well

preserved as judged by microscopic observation (see [31–33]).

For a more quantitative evaluation of the morphology of the chromosomes, integrated density profiles of a number of randomly selected hamster and human chromosomes were determined by digital image analysis. The integrated density profiles showed pronounced bimodal distributions and clearly detectable centromeric dips (for example see [35]).

The CIs for Chinese hamster chromosomes measured according to their PI fluorescence profiles were all compatible with the CIs calculated from a normal G-banded karyotype of the cell line used. For human chromosomes most of the CIs obtained by digital image analysis of their green-yellow fluorescence were found to fit well to the CIs of the human chromosomes X, 11 and 17 as determined by Lucas and Gray [8]. For Chinese hamster chromosomes a CI in the range of 0.7 was observed neither by us nor by Lucas and Gray [8]. It was compatible, however, with the CI of the human chromosome 17. We also calculated CIs of several interspecies translocations. These CI values varied between 0.56 and 0.73.

The density profiles underlined the good morphological quality of both labelled human chromosomes and non-labelled Chinese hamster chromosomes after the hybridization procedure under the conditions used.

Slit scan flow cytometry

Slit scan flow cytometry allowed us to select and to analyze suspension chromosomes which obviously had preserved their morphology under flow conditions after the hybridization procedure. Fig. 1 shows some typical examples of chromosome profiles (DAPI fluorescence intensity) after *in situ* hybridization, FITC labelling, and DAPI counterstaining. The shape of the profiles indicate a good morphology of the chromosomes and a correct alignment in the flow system.

Compared to conventional flow karyotyping, problems due to unspecific FITC fluorescence, which may lead to histogram signals underlying the chromosome peaks [3], could be considerably reduced by slit scanning. Software evaluation of different shape parameters for the profiles distinguished non-chromosomes and chromosomes as well as hybridized chromosomal material from FITC background. Only profiles having a significant (automatically detectable) centromeric dip were selected as fully intact chromosomes. This

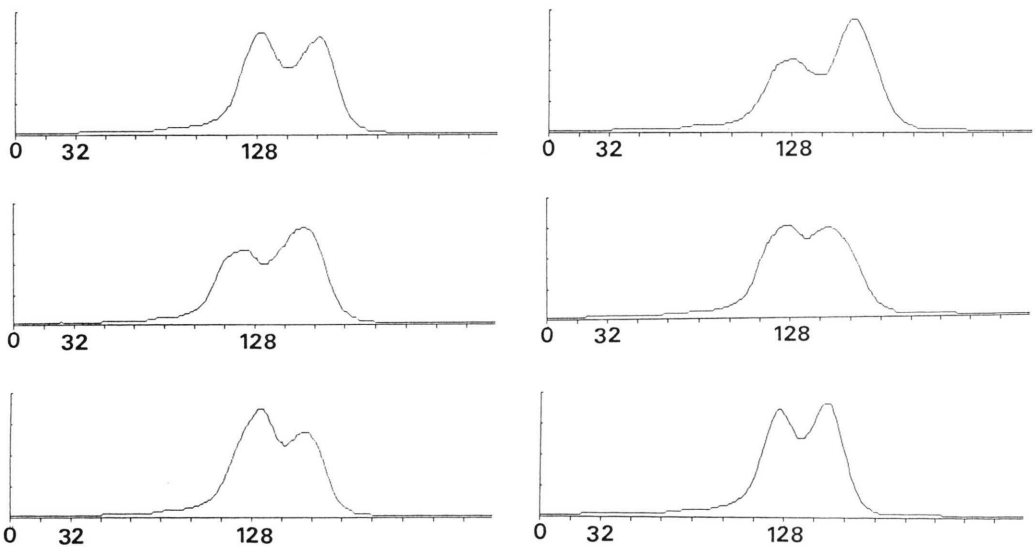

Fig. 1. Slit scan profiles of isolated chromosomes after *in situ* hybridization, FITC labelling, and counterstaining with DAPI (DAPI fluorescence intensity *vs.* time of flight in units of 100 nsec). The resolution of the slit scan system was about 3 μm. The different profiles are all gated to the same limits of integrated fluorescence intensities. Shown are six sequential bimodal profiles of a series, selected by this gating criterion, indicating a good chromosomal morphology under flow conditions and a correct alignment in the slit scan system.

means that together with non-chromosomes also chromosomes broken in the centromeric region as well as acrocentric chromosomes were excluded from consideration. In addition, according to this criterion also normal chromosomes were excluded which were not sufficiently well aligned in the flow system.

From 504 DAPI profiles measured from particles of a chromosome suspension after the *in situ* hybridization procedure, 98 (19.4%) were classified as normal chromosomes having a centromeric index of $0.5 \leq CI \leq 0.9$. This can be considered as the minimum amount of intact chromosomes under the conditions used. In this content, it may be mentioned that comparing the absolute lengths observed light microscopically, and the slit scan profile lengths of the chromosomes, a difference of about a factor of two could be estimated due to stretching of the chromosomes under flow conditions. This may be the reason that for control measurements of an aliquot of chromosomes of the same preparation but without *in situ* hybridization, the rate of fully intact chromosomes was only in the order of about 30% (data not shown).

Other two parameter slit scan experiments were performed using PI for counterstaining instead of DAPI (total number of profiles: 300; normal bimodal profiles: 17). Fig. 2 gives examples of measured profiles for a presumably normal human chromosome (A) and an interspecies translocation on the long chromosome arm (B). In both cases it is obvious that due to the PI profile (a) the morphology of the chromosome was well preserved and the alignment in the flow system was correct. The classification of the chromosomes resulted from the significant different distribution of the FITC fluorescence of the second profiles (b).

Chromosomes (characterized by their DAPI/PI profile) with a FITC distribution of high intensity correlating in profile length and showing a similar (bimodal) distribution as the DAPI/PI profile were interpreted to the labelled human chromosomes.

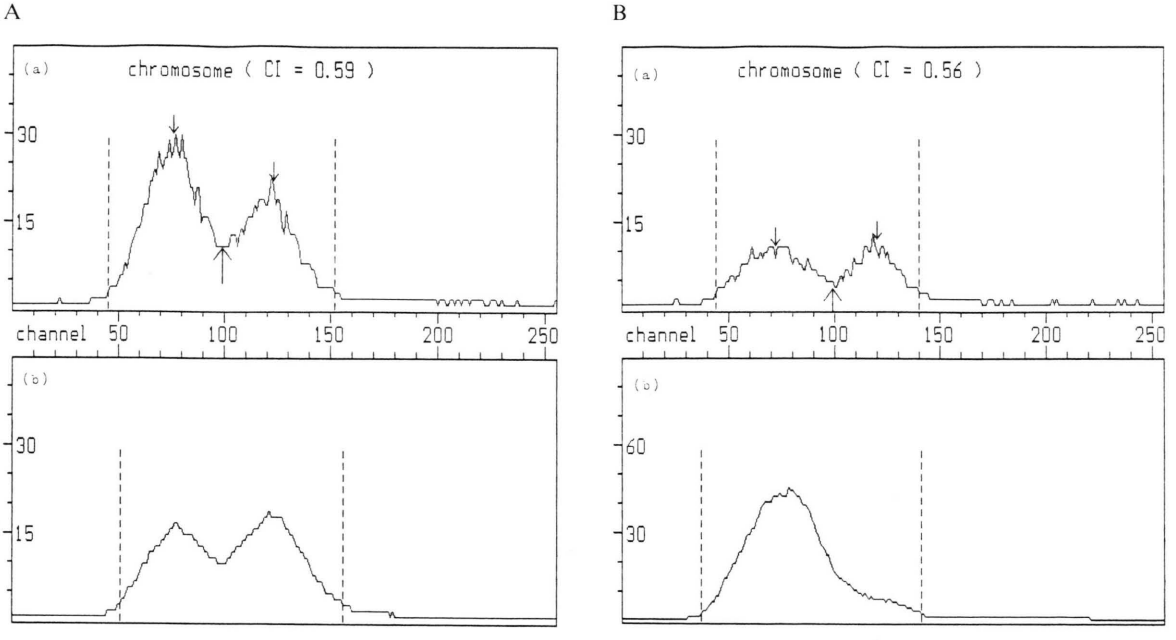

Fig. 2. Fluorescence intensities of PI (a) and FITC (b) *vs.* time of flight in units of 100 nsec (channel) measured by two parameter slit scan flow cytometry. The dashed lines indicate beginning and end of the chromosome profile determined by a rapid automated evaluation procedure (see text and Fig. 3). The centromere is automatically labelled by a large arrow (small arrows indicate positions of relative maxima). (A) Example of two slit scan profiles of one presumably normal human chromosome. Both profiles (a, b) indicate a normal bimodal distribution. (B) Example of two slit scan profiles of one presumably aberrant chromosome (interspecies translocation). The profiles show an uneven FITC fluorescence intensity (b) on the two arms of the chromosome (five times higher on the long arm than on the short arm) while the PI signal (a) indicates a normal bimodal fluorescence distribution.

Profiles with an "uneven" FITC distribution (see Fig. 2 B, b) suggest an uneven binding of the biotinylated DNA probe along the chromosomes. Such aberrant profiles were interpreted as interspecies translocation chromosomes.

Fig. 3 shows some examples of the automatic profile evaluation of two parameter measurements for a normal human chromosome (A) and a translocation chromosome (B). The first profile (a; DAPI counterstaining) shows the computed profile length, the CI, and the position of the centromere. According to several characteristics for the centromeric minima, a classification to chromosomes or non-chromosomes was obtained. The second profile (b: FITC labelling of the hybridized DNA) indicates the correlation of the FITC distribution to the chromosome length. If the intensity values of this profile were below a certain threshold (experimentally determined), the chromosome was classified as a non-hybridized Chinese hamster one. If not, the chromosome was considered to content human material. The third profile around the dotted line (c) resulted from the difference of the two other profiles normalized to the in-

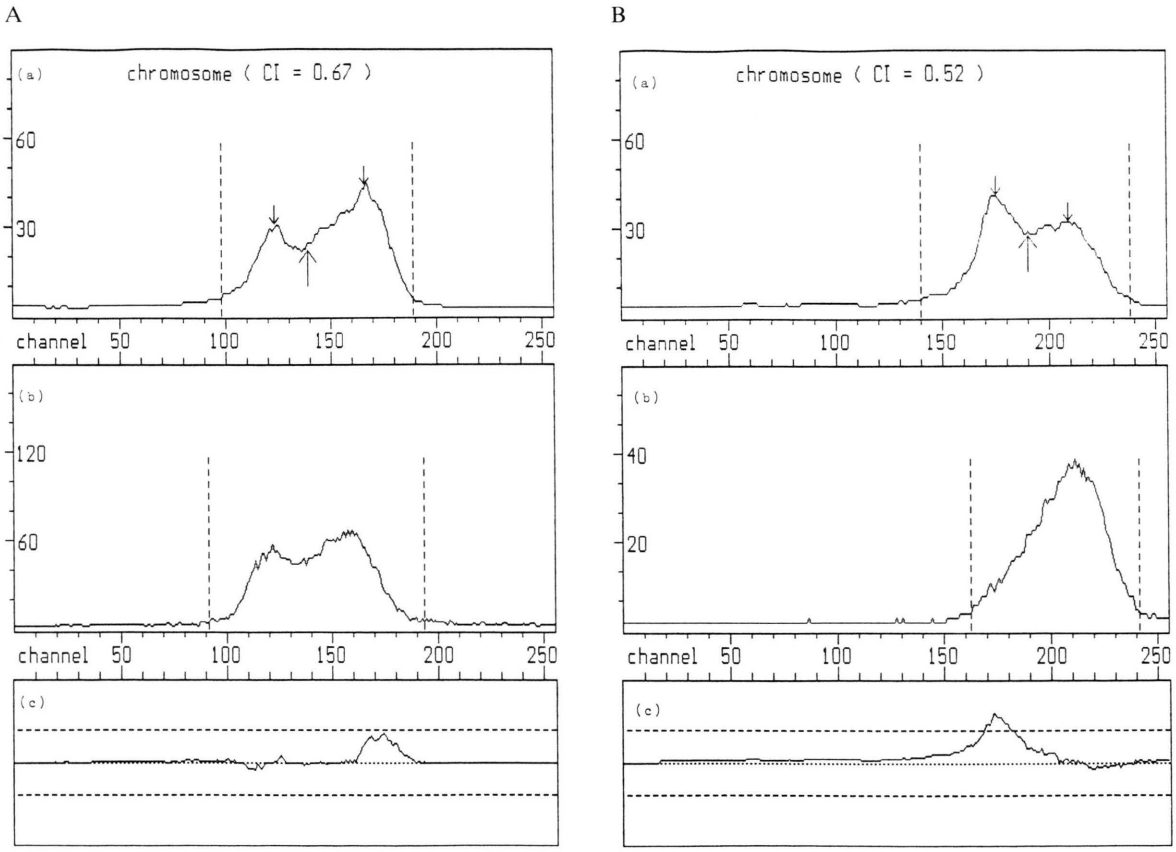

Fig. 3. Automated profile evaluation for a normal human chromosome (A) and a translocation chromosome (B). The profiles show the relative fluorescence intensity *vs.* time of flight in units of 100 nsec (channel). Profile (a) (DAPI counterstaining) shows the computed profile length (dashed lines), the CI, and the position of the centromere (large arrow). Profile (b) (FITC labelling of the hybridized DNA) indicates the correlation of the FITC distribution to the chromosome length (A: normal bimodal distribution; B: high intensity on the long chromosome arm). If the intensity values of this profile had been below a certain threshold (experimentally determined), the chromosome was classified as a non-hybridized Chinese hamster one. Profile (c) results from the difference of the two normalized profiles (a) and (b). If the values of this profile were between the thresholds indicated by dashed lines, the chromosome was classified as a normal human one. If the profile crossed the thresholds, the chromosome was classified as a translocation one. The entire time needed for the evaluation of the profiles and the classification ("normal", "translocation") was less than 3 msec classifying the two detected profiles and the difference profile.

tensity maximum. If the values of this profile were between the thresholds indicated by dashed lines, the chromosome was classified as a normal human one. If the profile crossed the thresholds, the chromosome was classified as a translocation.

An evaluation of the CIs of all selected DAPI slit scan profiles showed that the majority of them had CIs compatible either with the Chinese hamster chromosomes or with the human chromosomes in the cell line used. In particular, CI values were obtained compatible with the human chromosome 17 but not with any normal Chinese hamster chromosome of this cell line ($0.66 < CI < 0.74$). Additionally, all (except one) of these DAPI profiles associated with chromosome 17 showed a significant FITC profile as a second parameter. This was evaluated as normal human chromosomes and in two cases as interspecies translocations. The other human chromosomes of the cell line could also be detected by CI calculation and a significant FITC profile. The CIs of the DAPI slit scan profiles which correlated with aberrant FITC profiles agreed well with the CIs determined by digital image analysis for the interspecies translocations. The slit scan results obtained by computer evaluation are summarized in Fig. 4. The percentage of slit scan profiles assigned to Chinese hamster chromosomes (14.3%) was considerably lower than expected from the cytogenetic data (about 90%; see [33]).

A comparison with results from conventional flow karyotyping [3] revealed a high background due to broken chromosomes. A more detailed study of the background behaviour in flow karyotypes [42] indicated that larger chromosomes preferably break as compared to smaller ones. Since, in the cell line used, the human chromosomes have relatively small lengths, an over-representation after the hybridization procedure may be quite a reasonable result.

The results presented here show that flow cytometric detection of isolated chromosomes follow-

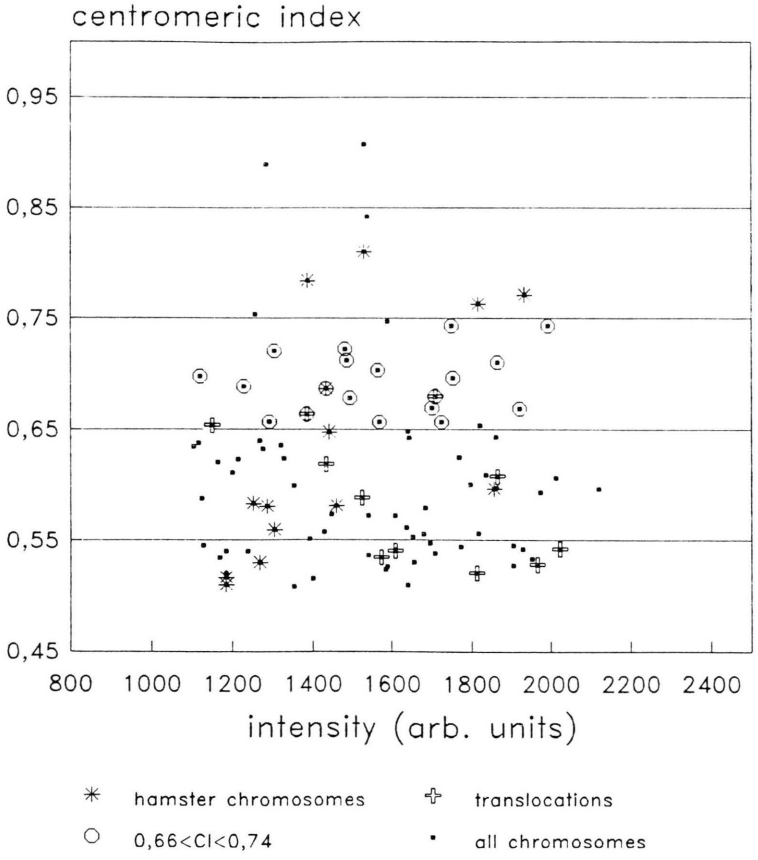

Fig. 4. Automated evaluation of the CIs of all DAPI slit scan profiles classified as chromosomes *vs.* the integrated fluorescence intensity in arb. units (for details see text).

ing fluorescence hybridization is indeed feasible. In particular, chromosomes displaying a well-preserved morphology can be selected and analyzed automatically in less than 3 msec. This means that on a PC a real time analysis of slit scan profiles (up to 100 per sec; see [2, 12]) may be feasible online. Eventually, the slit scan approach for translocation detection may lead to a fast aberration scoring. So far, chromosomes from a Chinese hamster hybrid cell line following fluorescence hybridization with genomic DNA have been used. The application to the detection of translocations and other chromosomal aberrations in human cells, however, requires optimized protocols for fluorescence *in situ* hybridization in suspension. The rapid progress in this field for chromosomes fixed on slides, especially the new methods of CISS hybridization (see introduction) suggests, however, that the development of such protocols will be possible in the near future.

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