Rapid Fluorescence In Situ Hybridization With Repetitive DNA Probes: Quantification by Digital Image Analysis[1]

Dino Celeda,[2] Klaus Aldinger, Frank-Martin Haar, Michael Hausmann, Markus Durm, Horst Ludwig, and Christoph Cremer[3]

Institute of Applied Physics (D.C., K.A., F.M.H., M.H., M.D., C.C.), Institute of Physical Chemistry (M.D., H.L.) and Interdisciplinary Centre of Scientific Computing (IWR) (C.C.), University of Heidelberg, Heidelberg, Germany

Fluorescence in situ hybridization (FISH) has become an important tool not only in cytogenetic research but also in routine clinical chromosome diagnostics. Here, results of a quantification of fluorescence signals after in situ hybridization with repetitive DNA probes are reported using a non-enzymatic hybridization technique working with a buffer system not containing any fonnamide or equivalent chemical denaturing agents. Following simultaneous denaturation of both cells and DNA probes, the renaturation time was reduced to less than 30 min. For one of the DNA probes reasonable FISH-signals were even achieved after about 30 s renaturation time. In addition, the number of washing steps was reduced drastically. As a model system, two repetitive DNA probes (pUC 1.77, D15Z1) were hybridized to human metaphase spreads and interphase nuclei obtained from peripheral blood lymphocytes. The probes were labelled with digoxigenin and detected by FITC-anti- digoxigenin. The hybridization time was reduced step by step and the resulting fluorescence signals were examined sys-tematically. For comparison the pUC 1.77 probe was also hybridized according to a FISH protocol containing 50% formamide. By renaturation for 2 h and overnight two FISH signals per nucleus were obtained. Using shorter renaturation times, no detectable FISH signals were observed. Quantification of the FISH signals was performed using a fluorescence microscope equipped with a cooled colour charge coupled device (CCD) cam-era. Image analysis was made interactively using a commercially available software package running on a PC (80486). For the pUC 1.77 probe the major binding sites (presumptive chromosomes 1) were clearly distinguished from the minor binding sites by means of the integrated fluorescence intensity. For the two (pUC 1.77) or four (D15Z1) brightest spots on the metaphase spreads and in the interphase nuclei hybridized without formamide, integrated fluorescence intensity distributions were measured for different renaturation times (0.5, 15, 30 min). The intra-nuclear variation in the intensity of the two brightest in situ hybridization spots appeared to be slightly higher (CV between 16 and 32%) than the corresponding variation on the metaphase spreads (CV between 10 and 19%). For the D15Z1 probe FISH signals were detected after hybridization without formamide and 15 min and 30 min renaturation. Always four bright spots were visible and tentatively assigned on the metaphase spreads (presumptive chromosome 15 and 9). The intensity variation of each pair of homologues in a metaphase spread showed a CV of 14 or 15%, respectively, for the presumptive chromosome 15, and 8 or 9%, respectively, for the presumptive chromosome 9. © 1994 Wiley-Uss, Inc.

Key terms: Rapid fluorescence in situ hybridization, quantification, colour CCD camera, image analysis, repetitive DNA probes

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[2] Dino Celeda's present address is Institute for Molecular Biotechnology e.V., D-07745 Jena, Germany.

[3]Address reprint requests to Dr. Christoph Cremer, Institut für Angewandte Physik, Albert-Überle-Str. 3-5, D-69120 Heidelberg, Germany.

Fluorescence in situ hybridization (FISH) (3, 14, 38, 53, 57) allows the detection of specific DNA and RNA sequences at the individual cell level. It has become an important tool for chromosomal research, cell biology, and cytogenetic diagnostics (10, 42, 45, 60, 61). During the last few years the sensitivity of FISH has improved considerably. It now allows the detection of only a few kilobases (17, 33, 40, 44, 55) up to the delineation of whole chromosomes (15, 22, 43, 52, 54, 58) using complex DNA probes established from flow sorted chromosomes (12, 19, 64). Besides the improved sensitivity,multi colour FISH-protocols were developed for the simultaneous detection of different chromosomes and chromosomal regions (22, 32, 34, 47, 51, 56).

All over the world cytogeneticists established FISH-protocols which have been routinely used in their laboratories. More and more those protocols became important for clinical applications (1, 14, 18, 20, 26, 37, 60). However, in the case of clinical routine it would be desirable to accelerate, and to automatize the hybridization process.

A useful step to realize these demands was the introduction of fully automatized polymerase chain reaction (PCR) techniques (46) for probe amplification for FISH (23, 41, 65). This was also done in combination with direct incorporation of labelled nucleotides (8, 66) so that nick translation became largely unnecessary.

Recently, an interesting alternative to classical FISH has been introduced called PRINS (primed in situ labelling) (35, 36). It is a one-step procedure based on the sequence specific annealing of unlabelled DNA to the chromosomal target DNA in situ. The unlabelled specific DNA then serves as primer and the target as template to synthesize DNA directly on the chromosome. Using labelled desoxyribonucleotides as substrates for this purpose, the relevant sequences can be visualized. Compared to FISH, PRINS eliminates the need for separate probe labelling. Using much higher probe concentrations, PRINS was considerably faster than classical FISH for the same test DNA probes (25).

The PRINS technique may be easily automatized. A disadvantage, however, may be the high amount of DNA-probe and substrate to achieve reasonable signals after a time of at least 30 min. Moreover, this resulted in fluorescence signals which at the most had only the same intensities as classical FISH. Thus, especially in routine applications, sufficiently accelerated FISH protocols may compliment the PRINS technique. From the very early reports published on FISH of DNA probes most laboratories use high quantities of formamide (about 40-65%) or equivalent chemical denaturing agents in the hybridization buffer in order to increase the efficiency. Consequently, a number of different more or less time-consuming washing steps have to be done after the hybridization to wash out those chemicals. Here, we describe systematic, quantitative studies on an alternative, rapid, non-enzymatic hybridization method that avoids the use of any formamide or equivalent chemical denaturing agents, and conse-

quently reduces the number of necessary washing steps drastically. This technique is based on protocols recently published (7).

So far, most of the more than one thousand publications on FISH dealt with the qualitative description of he hybridization results. Quantification was usually restricted to counting of spots, to the determination of spot distances in nuclei, or to the enumeration of aberrations (e.g., 16, 20, 24, 31, 54). Recently, more quantitative studies concerning the fluorescence contents of FISH signals were published (22, 48, 50). They applied the developments of digital image microscopy with black and white CCD cameras (2, 30, 49). Here, we used a cooled colour CCD-camera on a fluorescence microscope (Leitz Orthoplan). To obtain quantitative data from these FISH experiments, as a model system human peripheral blood lymphocytes hybridized with repetitive DNA probes were taken. The digitized FISH-signals were evaluated using a commercially available imaging program. Since the optimal instrumentation setups are not independent from the optimal hybridization protocol and vice versa, such commercially available components may offer a certain standard for comparison of fluorescence results of different hybridization techniques.

MATERIALS AND METHODS
Slide Preparation

Metaphase chromosomes and cell nuclei were obtained from human lymphocytes isolated from peripheral blood by standard techniques (4). The lymphocytes were stimulated by Phytohemagglutinin M (2.5 (µg/ml lymphocyte medium) and cultivated for 72 h followed by a Colcemid block (27 (xM) (Boehringer Mannheim, Mannheim, Germany) of 2 h. The cells were treated according to a modified hexandiol method (21) and the metaphase spreads and interphase nuclei were fixed on slides by means of methanol/acetic acid (3:1, v/v) (57).

Preparation of DNA Probes

For the pUC 1.77 DNA probe (1.77 kb) cloned in a pUC 9 vector (major binding site: constitutive heterochromatin of human chromosome 1) (9) and for the D15Z1 DNA probe (1.8 kb) cloned in a pUC 8 vector (major binding site: centromeric region of human chromosome 15) (59), entire plasmids (kindly provided by Prof. Dr. T. Cremer, Institute of Human Genetics and Anthropology, University of Heidelberg) with the human insert were labelled with Digoxigenin-11-dUTP by nick translation (Nick Translation Kit, Boehringer Mannheim, Germany) according to product information.

In Situ Hybridization Without Formamide

In situ hybridization without formamide or equivalent chemical denaturing agents (referred to as without formamide) was performed as described in detail else- where (7). Briefly: Approximately 70 ng of the labelledDNA probe, 3 µl hybridization buffer (10 x: Tris-HCl

100 mmol/1; MgCl$_2$ 30 mmol/1; KCl 500 mmol/l; gelatine 100 µg/ml; pH 8.3 [20°C]), and 3 µl20 x SSC were diluted in deionized HgO to make up a final volume of 30 µl. This hybridization mixture was pipetted on the microscope slides with the fixed metaphase spreads and nuclei. The slides were covered with a cover glass, sealed with rubber cement (Fixogum, Marabu, Tamm, Germany), and placed in a specially designed, closed stainless steel chamber.

Thermal denaturation was performed at 94°C for 5 min. This denaturation temperature was estimated from hyperchromicity curves registered at a wavelength of 256.6 nm for human lymphocyte metaphase chromosomes and the pUC 1.77 probe in the hybridization buffer used (Adam et al., unpublished results). For the pUC 1.77 probe, changes in the hyperchromicity due to denaturation were registered below 80°C. For the chromosome suspension, the curve suggested a melting point of about 85°C. The change of extinction was found to be finished at about 90°C. Thus, a denaturation temperature of 94°C appeared to be acceptable.

For renaturation the steel chamber with the slides was placed into a water bath of 40°C for 30 min, or 15 min, respectively. For the experiments with the shortest renaturation time (about 30 s) the slides were removed from the steel chamber immediately after denaturation and processed as described below.

In Situ Hybridization With Formamide

In situ hybridization was also performed using formamide in a modified hybridization buffer as described above, which was compatible to standard protocols (e.g., 63): Briefly: Approximately 70 ng of the labelled DNA probe (pUC 1.77), 15 µl deionized formamide (= 50% final concentration), and 3 µl 20 x SSC were diluted in H$_2$O to make up a final volume of 30 µl. This hybridization mixture was pipetted on the microscope slides with fixed metaphase spreads. They were covered with a cover glass and sealed with rubber cement (Fixogum, Marabu, Tamm, Germany). The slides were also placed in the steel chamber. Thermal denaturation was performed at 75°C for 5 min. For renaturation the steel chamber with the slides was placed into a water bath of 40°C for 5 min, 30 min, 120 min, and overnight. After renaturation the cover glasses were removed and the slides were washed three times for 5 min at room temperature in prewarmed (45°C) deionized formamide/2 x SSC (1:1; pH 7.0). Then they were washed three times for 5 min at room temperature in pre-warmed 0.1 x SSC (60°C).

Detection

For detection, the slides were washed in a solution of 0.9% NaCl/0.2% Tween 20 for 5 min at room temperature and then air dried. For fluorescence labelling with antidigoxigenin-fluorescein Fab fragments (Boehringer Mannheim, Germany) the stock solution (200 µg/ml) was diluted in 0.9% NaCl/0.2% Tween 20 to a final

concentration of 2 µg/ml. Approximately 70 µl of this solution was pipetted on each slide, which was covered with a cover glass and again sealed with rubber cement. The slides were replaced into the steel chambers and incubated for 2 h at 37°C. No blocking step was performed. After incubation the slides were washed again in 0.9% NaCl/0.2% Tween 20 for 5 min in the dark. For counterstaining of the chromosomes, propidium iodide (PI) (5 µmol/1 or 15 µmol/l, respectively) or diamidinophenylindole (DAPI) (5 µmol/l) were used.

Microscopy and Digital Image Analysis

For visualization, a fluorescence microscope (Leitz Orthoplan) was used equipped with an objective PlanAPO 63 x /NA 1.40 and a 50 W mercury arc lamp.
With

$$D = 2\lambda / [4n (1- (1-(NA/n)^2)^{1/2})]$$

[λ = excitation wavelength; n = refractive index of the immersion oil; NA = numerical aperture of the objective]

the depth of field D was calculated to be about 0.25 µm. This formula is compatible to the assumption of a Struve ratio of the OTF of 0.86 corresponding to the diffraction limit of the lens (68). For practical reasons (visible sharpness of the images) a Struve ratio of 0.5, which is compatible to the full-width half-maximum of the intensity distribution around the focus, appeared to be tolerable (6). This resulted in a depth of field of about 0.75 p.m. Excitation took place via a band pass filter (450-490 nm) and detection via a 515 nm long pass filter. The images of all metaphase spreads or interphase nuclei in a given area with two or more hybridization signals were registered by a cooled colour CCD camera (CF 15 MC, Kappa, Gleichen, Germany) having an interline CCD chip with integrated mosaic filter and a resolution of 460 x 440 pixels. The images were transferred to a colour frame grabber (ITI Vision + Color CFG 512) with 28 bit (8 bit red, 8 bit green, 8 bit blue, 4 bit overlay). For registration and interactive evaluation, the commercially available software package OPTIMAS running on a PC (80486) under WINDOWS 3.1 with the operating system MS-DOS was used.

For all images a constant acquisition time of 9 s was chosen to achieve an optimum intensity distribution over the 8 bit of the green image. The typical depth of an image was about 6 to 7 bit on average. For the image display, a one to one ramp look up table was used. This means that a light intensity value (shade of gray in one colour plane) that entered passed out as an identical light intensity value. The digitized images were visualized without any further image processing (e.g., filtering, contrast enhancing operations, or background corrections).

All quantitative results were obtained from the green image. For interphase nuclei, integrated intensity profiles were registered along a line through the two brightest spots and through the two spots with the next lower integrated intensity (Fig. 1a). The width of
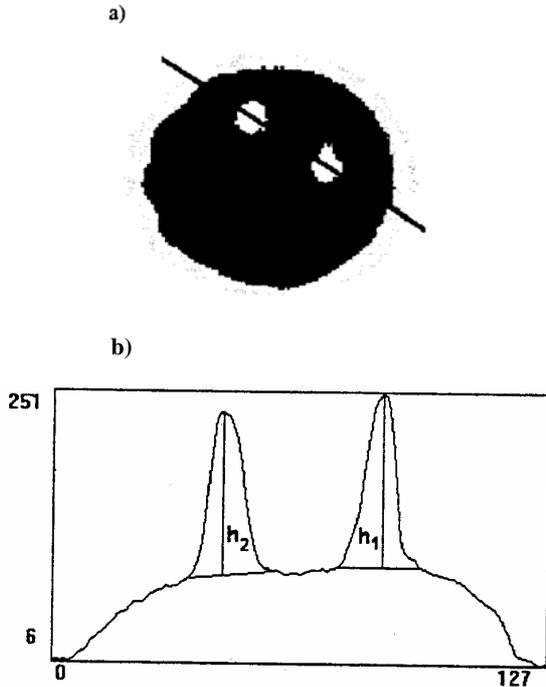
a)

b)

FIG. 1. Representation of the analysis procedure for nuclei, (a) integration line through the hybridization spots, (b) determination of the maximum intensity h_1 and h_2 from the integrated intensity profile after interactive background subtraction. Ordinate: integrated intensity in arbitrary units (a.u.); abscissa: normalized length in a.u.

this line (4-20 pixels) was chosen in such a way that the width of the spots was entirely covered. From the integrated intensity profiles of these spots the intensity maxima h, (i = 1,..., 4) were determined in arbitrary units (a.u.) after interactive linear background subtraction (Fig. Ib). In the following, these maxima of the integrated intensity profiles will be referred to as "intensities." For the metaphase spreads, the integration lines were positioned along the chromosome axes with the two, or, where possible four, brightest hybridization signals (Fig. 2a). The intensities were determined from the maximum of the integrated intensity profiles in the same way as for the interphase nuclei (Fig. 2b). For h_1/h_2, and h_3/h_4, $h_1 + h_2$ and $h_3 + h_4$, as well as $(h_1 + h_2)/(h_3 + h_4)$, the mean value μ, the standard deviation σ and the coefficient of variation CV = 100% * σ/μ, were calculated. Fluorescence signals were evaluated only if they clearly exceeded the background level(about 10 a.u.). In addition, the spot sizes were determined in pixel numbers.

RESULTS

Metaphase chromosomes and interphase nuclei of human lymphocytes were hybridized with two repetitive DNA probes (pUC 1.77, D15Z1) using a hybridiza-

tion technique that omits the use of formamide or equivalent chemical denaturing agents. The influence of three different renaturation times t_n (30 min, 15 min, about 30 s) on the fluorescence signals of major and minor binding sites was examined. In the cases of t_n = 30 min and t_n = 15 min, the major binding sites and several minor binding sites were visible for both probes. In the case of t_n = 30 s, hybridization signals were only obtained for the pUC 1.77 probe. In all experiments with a renaturation time of t_n = 30 min, the number of visible minor binding sites per cell appeared to be higher than in the experiments with t_n = 15 min renaturation time (Table 1).

For comparison, FISH was also performed using a protocol containing formamide in the hybridization buffer. For these experiments only the pUC 1.77 probe was used. For t_n = 15 min and t_n = 30 min, no detectable FISH signals were found in cell nuclei or on the longest chromosomes of the metaphase spreads. After t_n = 120 min as well as after renaturation overnight, always two bright FISH signals were observed. In Figure 3a,b, 20 intensity profiles of the longest four chromosomes (presumptive chromosomes 1 and 2) of 5 metaphase spreads normalized to the same chromosome length and equal centromere position were integrated and averaged. This was done for the experiments with t_n = 30 min using both the hybridization protocol with and without formamide. The profile from metaphase chromosomes hybridized with formamide (Fig. 3a) showed a relatively homogeneous intensity distribution only. In contrast, the profile from metaphase chromosomes hybridized without formamide or equivalent denaturing chemical agents (Fig. 3b) clearly showed a considerably increased fluorescence intensity in the centromeric region (although the scale of the ordinate has been reduced by a factor 5). This is well compatible with the position (1q12) of the pUC 1.77 probe in the subcentromeric region of the constitutive heterochromatin of chromosome 1. The profile shape of Figure 3b is in good accordance to Figure 3c, where normalized integrated intensity profiles of 20 chromosomes with fluorescence signals after FISH with formamide t_n = 120 min) were integrated and averaged $(h_{withoutform, 30 min}/h_{with form, 120 min} = 3.96)$.

In all following experiments, hybridization was always performed using the protocol without formamide or equivalent denaturing chemical agents: For the pUC 1.77 probe two hybridization regions of high intensity were always visible. On the metaphase spreads, the brightest spots were localized on the longest chromosomes compatible with the assignment of this probe to chromosome 1 (9). Besides those two spots, several spots with considerably lower fluorescence intensity were detected, which in all cases were located on medium sized or small chromosomes. These spots were interpreted as the minor binding sites of the DNA-probe. Chromosome length and centromeric indices were compatible with locations on chromosome 9 16 or Y (27).
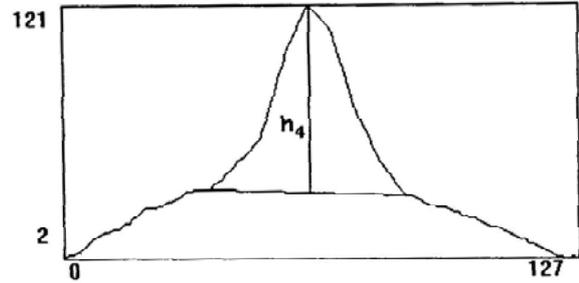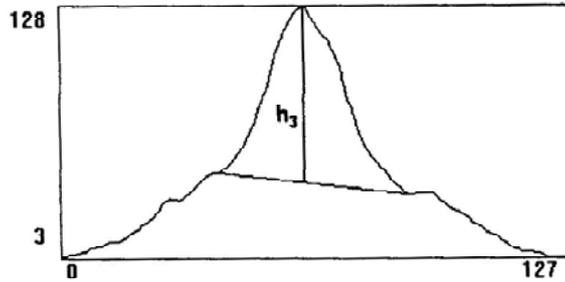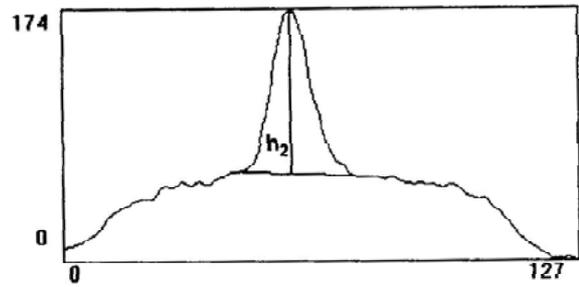
a)
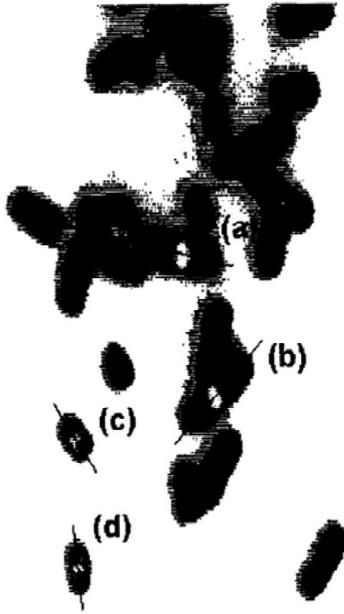
b)

chromosome (a)
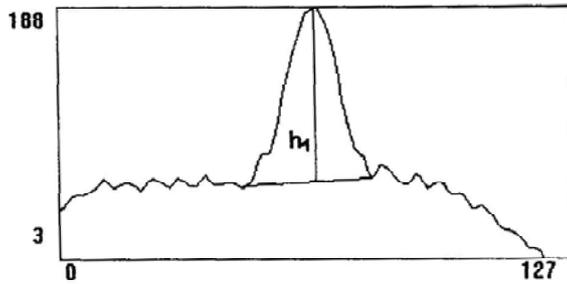
chromosome (b)

chromosome (c)

chromosome (d)

FIG. 2. Representation of the analysis procedure for metaphase spreads, (a) integration lines through the metaphase chromosomes with spots, (b) determination of the maximum intensities h_1, h_2, h_3, h_4 of the chromosome regions with spots from the integrated intensity profiles after interactive background subtraction. Ordinate: integrated intensity in arbitrary units (a.u.); abscissa: normalized length in a.u.

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Table 1
Mean Number of Minor Binding Sites Per Nucleus and Per Metaphase Spread, Respectively, With a Signal Intensity Higher Than Background Fluctuations[a]

	30 sec	15 min	30 min
pUC 1.77 nuclei	0.68 (N = 36)	0.60 (N = 32)	0.68 (N = 38)
pUC 1.77 metaphase spreads	0.30 (N = 13)	1.06 (N = 15)	1.23 (N = 26)
D15Z1 metaphase spreads		7.80 (N = 19)	11.4 (N = 18)

[a]In metaphase spreads, all hybridization spots above background were counted except the signals from the presumptive chromosome 1 (pUC 1.77 probe) and from the presumptive chromosome 9 and 15 (D15Z1 probe), respectively. In nuclei following FISH with the pUC 1.77 probe, the signals from the major binding sites were assumed to be the two brightest ones.

Hybridization with the D15Z1 probe showed always four spots of high fluorescence intensity. With this probe, reasonable FISH signals were achieved for renaturation times of t_n = 15 min and t_n = 30 min (30 s renaturation time gave no detectable results). In metaphase spreads, the spots of high intensity were registered on chromosomes of types "A" and "B." From the chromosome length and the centromeric index, chromosomes type "A" were compatible with chromosome 15, to which the probe should be specific under high stringency conditions. Chromosomes type "B" were compatible with chromosome 9, which is known as a minor binding site of the D15Z1 probe under high stringency conditions (29). For the D15Z1 probe, only metaphase spreads were evaluated. In the nuclei the spot intensities were slightly different from the metaphases. The four spots in each nucleus did not show a significant difference in their intensities. Therefore tentative identification and assignment (discrimination of chromosome 9 or 15) was not feasible.

Figure 4 shows the variation of the spot intensities on the two homologous metaphase chromosomes after hybridization with the pUC 1.77 probe and three different renaturation times (t_n = 30 min, 15 min, 30 s). For each metaphase spread the intensity of the spot signal with the second highest intensity *(h_2)* was plotted as a function of the spot signal with the highest intensity (h_1).In Figure 5 the same dependence is given for the evaluated cell nuclei. Linear regression lines are shown for Figures 4 and 5. In Table 2 the results of the two figures are summarized. The intranuclear variation (CV of $\mu[h_1/h_2]$ between 16 and 32%) in the intensity of the two hybridization spots appeared to be higher than the corresponding variation on the metaphase spreads (CV of $\mu[h_1/h_2]$ between 10 and 19%). The inter-nuclear variation showed a CV of $\mu[h_1 + h_2]$ between 46 and 60%. The corresponding value for the metaphase homologues was between 30 and 46%. A clear correlation to the renaturation time was
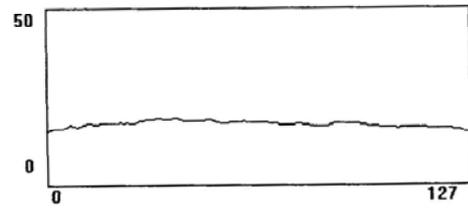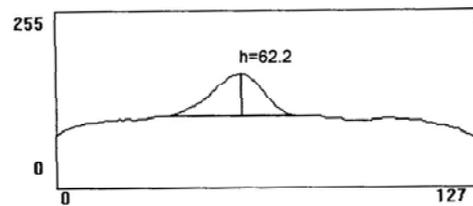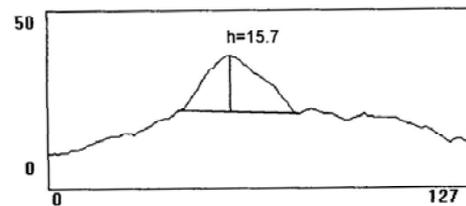

FIG. 3. Integrated intensity profiles after normalization to chromosome length and averaging, (a) application of a hybridization protocol containing formamide in the hybridization buffer (t_n = 30 min); (b) application of a hybridization protocol containing no formamide or equivalent denaturing chemical agents in hybridization buffer (t_n = 30 min); (c) application of a hybridization protocol containing formamide in the hybridization buffer (t_n = 120 min). Twenty orientated(telomere of the short arm at position 0) intensity profiles normalized to the chromosome length of 127 a.u. were integrated-and averaged. In (a) and (b) the four longest chromosomes (presumptive chromosome 1 and 2) of a metaphase spread were taken independently from a visible FISH signal. In (c) only the two longest chromosomes (presumptive chromosome 1) with a visible FISH signal were taken. The scale of the ordinate of (b) is reduced by a factor 5.

not apparent. Similar results concerning linear regression curves were obtained by analyzing the spot intensities of the two next less intensive minor binding sites (data not shown).

Figure 6 shows the variation of the sum of the spot intensities $h_3 + h_4$ presumptive chromosome 9) as a function of the spot intensities $h_1 + h_2$ (presumptive chromosome 15). The gradient of the regression line in Figure 6a (t_n = 30 min) was reduced compared to Fig-

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ure 6b (t_n = 15 min). For 15 min renaturation time the intensities (h_1, h_2) of the spots on chromosome "A" (presumptive chromosome 15) were similar to the intensities (h_3, h_4) of the spots on chromosome "B" (presumptive chromosome 9) of the same metaphase spread [$\mu(h_1 + h_2/h_3 + h_4) = 0.99 \pm 0.09$]. For 30 min renaturation time, however, the intensities on chromosome "A" were always significantly lower [$\mu(h_1 + h_2/h_3 + h_4) = 0.58 \pm 0.10$] than on the corresponding chromosome "B" (Table 3).

Figure 7 shows the variation of the spot intensities on the two homologous metaphase chromosome pairs after hybridization with the DI5Zl probe. For both renaturation times the CVs had similar values. The CV of $\mu(h_3/h_4)$ corresponding to the presumptive chromosome 9 (8% for t_n = 30 min; 9% for t_n = 15 min) appeared to be even smaller than the CV of $\mu(h_1/h_2)$ of the presumptive chromosome 15 (14% for t_n = 30 min; 15% for t_n = 15 min). The variation between different metaphase spreads was about the same for both chromosome types in the case of t_n = 15 min [CV($\mu(h_1 + h_2)$) = 27%; CV($\mu(h_3 + h_4)$)= 28%]; in the case of t_n = 30 min the CV of $\mu(h_3 + h_4)$ was observed to be considerably smaller (= 23%) than the CV of $\mu(h_1 + h_2)$ (= 36%). In Table 3 the results for the D15Z1 probe are summarized.

As an additional parameter to classify the hybridization spots, the spot area can be used. In Table 4 the results are summarized for the mean values. In the case of the pUC 1.77 probe the spot sizes of the major binding sites on the metaphase spreads differ from the spot sizes of the minor binding sites within one standard deviation. The evaluation of the nuclei confirm this result. Besides two large spots several smaller spots were detected. For the D15Z1 probe similiar results (metaphase spreads) were obtained, although the areas of the major binding sites are smaller than the minor binding sites on chromosome 9.

DISCUSSION

To simplify the FISH procedure for specific labelling of chromosome regions with repetitive DNA probes, we have developed a non-enzymatic technique working without any formamide or equivalent denaturing chemical agents. Thus the number of time-consuming washing steps after hybridization was reduced to two for the entire procedure. Due to the lack of formamide or equivalent denaturing chemical agents in the hybridization buffer, the renaturation time required for reasonable fluorescence signal intensity was reduced to a minimum of 30 s compared to 2 h or overnight, respectively, using a formamide protocol. Thus the entire hybridization procedure can be performed in a minimum of about 15-30 min. Nick translation is not absolutely necessary if one incorporates labelled nucleotides by PCR (8, 41, 46, 65), which can be fully automated. Using directly fluorochromized nucleotides (meanwhile commercially available) may avoid the incubation time necessary for the detection by im-
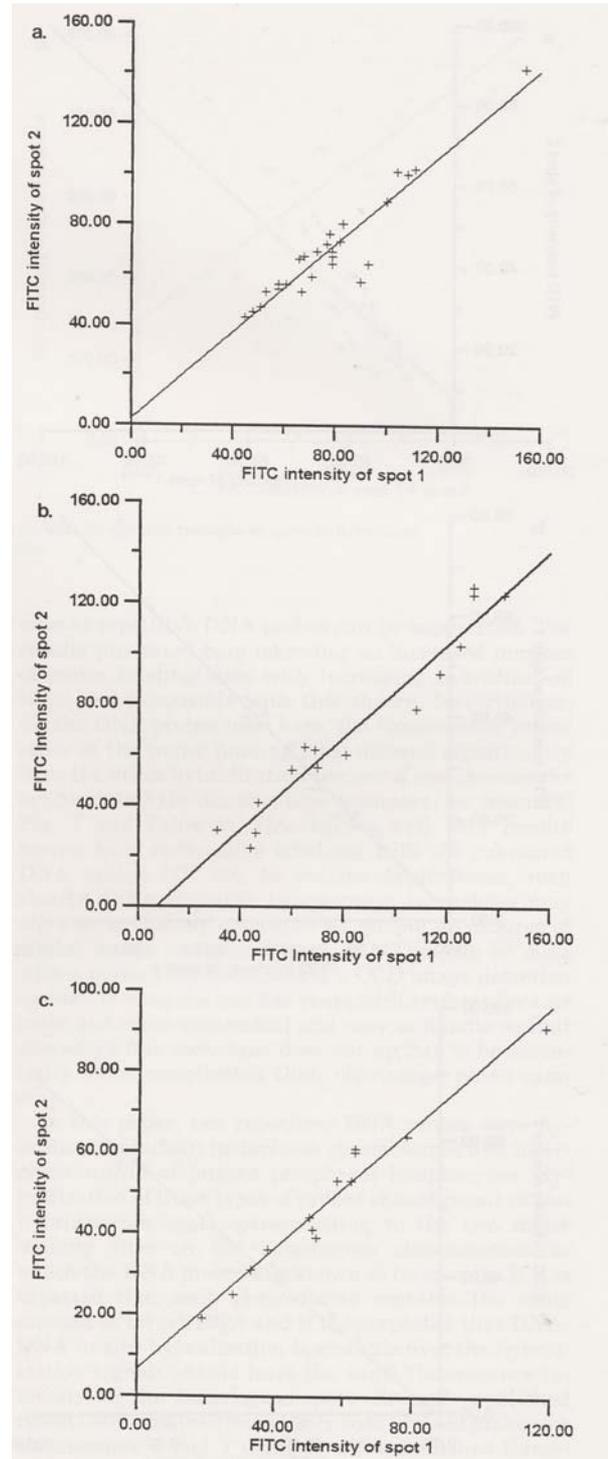

FIG. 4. Intensities h_2 vs. h_1 (in arbitrary units) for spots in metaphase spreads hybridized with the pUC 1.77 probe (**a**) t_n= 30 min (**b**) t_n = 15 min, (**c**) t_n = 30 s.
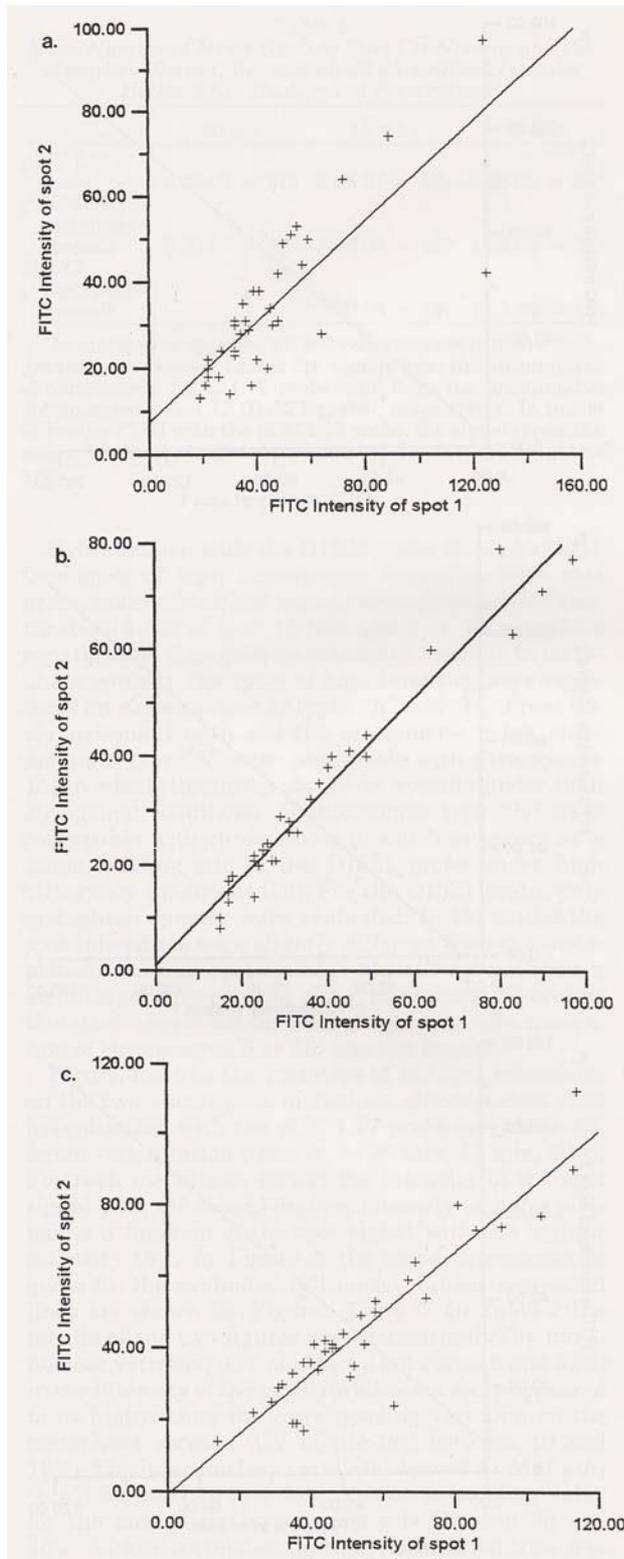
FIG. 5. Intensities h_2 vs. h_1 (in arbitrary units) for spots in interphase nuclei hybridized with the pUC 1.77 probe, (a) t_n = 30 min (b)t_n = 15 min, (c) t_n = 30 s.

Table 2
Evaluation of the Hybridization Spots on Metaphase Spreads and Interphase Nuclei After FISH With pUC 1.77 for Different Renaturation Times[a]

pUC 1.77	30 sec	15 min	30 min
Number of metaphase spreads	13	15	26
Mean $\mu(h_1)$	54.85	84.33	78.30
Mean $\mu(h_2)$	47.46	70.80	70.15
S.D. $\sigma(h_1)$	25.05	37.35	23.84
S.D. $\sigma(h_2)$	19.18	35.26	21.99
$\mu(h_1/h_2)$	1.14	1.24	1.12
$\sigma(h_1/h_2)$	0.11	0.24	0.14
Coefficient of variation CV	10%	19%	12%
$\mu(h_1 + h_2)$	102.31	155.13	148.46
$\sigma(h_1 + h_2)$	44.00	72.04	45.14
CV	43%	46%	30%
Number of interphase nuclei	36	32	38
Mean $\mu(h_1)$	53.86	36.88	43.97
Mean $\mu(h_2)$	44.56	31.84	33.05
S.D. $\sigma(h_1)$	24.24	22.55	24.59
S.D. $\sigma(h_2)$	22.03	19.20	17.77
$\mu(h_1/h_2)$	1.23	1.19	1.39
$\sigma(h_1/h_2)$	0.28	0.19	0.45
Coefficient of variation CV	23%	16%	32%
$\mu(h_1 + h_2)$	98.14	68.72	77.13
$\sigma(h_1 + h_2)$	45.50	41.53	40.59
CV	46%	60%	53%

[a] μ = mean; σ = standard deviation; h_1, h_2 = intensity maximum 1, 2; CV = $(\sigma/\mu)* 100$ %. All values except CV are given in arbitrary units.

munofluorescence and eliminate another washing step.

In principle, in situ hybridization signals after application of a formamide protocol can also be detected in a relatively short time (about 10 min) (39). But in these cases ^{32}P radioactive labelled DNA probes were used. For an immediate quantification, Cerenkov counts (β- radiation of 1.71 *MeV;* specific activity 0.8-2.2 x 10^8 cpm/μg DNA probe) were measured by a scintillation counter. Although this method was fast and sensitive, it did not give any information about the cellular *distribution* of the ^{32}P-sources, in contrast to fluorescence microscopy, which gives a direct local correlation of the FISH signal and the hybridized chromosome region. The spatial resolution using radioactive isotopes might of course be enhanced considerably by using, e.g., ^{3}H- labelled nucleotides. This perhaps might allow a similar reduction in renaturation time; however, the time required for detection (autoradiography) would be increased by orders of magnitude. This disadvantage includes several problems in handling radioactive DNA probes. Here, we have introduced an alternative nonradioactive method which combines the advantages of hybridization speed (short renaturation times), spatial resolution in detection, and fast registration (FISH procedure).

Compared to, the PRINS-technique (35, 36), the FISH procedure described here can be performed in about the same time having a similar technical equipment. It may be pointed out, however, that according to the lit-
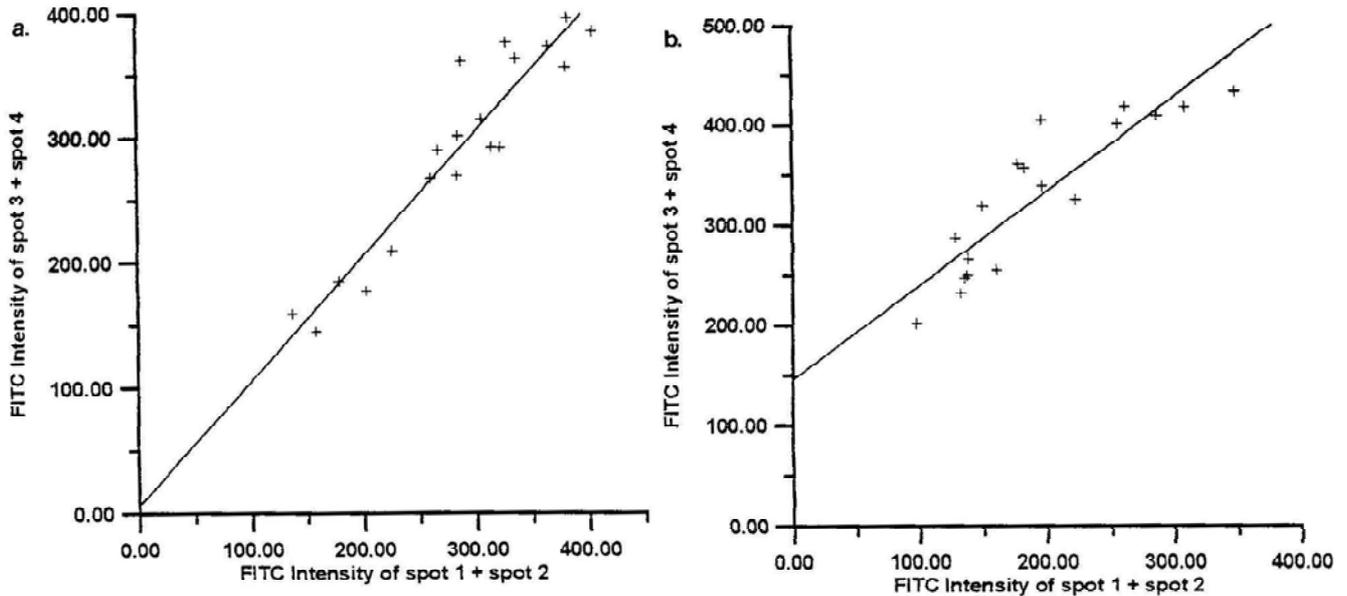
FIG. 6. Intensities $h_3 + h_4$ vs. h_1; $+ h_2$ (in arbitrary units) for spots in metaphase spreads hybridized with the D15Z1 probe, (a) $t_n = 30$ min, (b)$t_n = 15$ min.

Table 3

Evaluation of the Hybridization Spots on Metaphase Spreads After FISH with D15Z1 for Two Different Renaturation Times[a]

D15Z1	15 min	30 min
No. of metaphase spreads	19	18
Mean $\mu(h_1)$	152.24	109.17
Mean $\mu(h_2)$	133.61	86.61
S.D. $\sigma(h_1)$	38.21	36.94
S.D. $\sigma(h_2)$	40.20	34.58
$\mu(h_1/h_2)$	1.77	1.30
$\sigma(h_1/h_2)$	0.17	0.18
CV	15%	14%
$\mu(h_3/h_4)$	1.13	1.19
$\sigma(h_3/h_4)$	0.10	0.10
CV	9%	8%
$\mu(h_1 + h_2)$	286.05	195.78
$\sigma(h_1 + h_2)$	77.29	70.69
CV	27%	36%
$\mu(h_3 + h_4)$	290.00	329.00
$\sigma(h_3 + h_4)$	81.48	74.90
CV	28%	23%
$\mu(h_1 + h_2/h_3\ h_4)$	0.99	0.58
$\sigma(h_1 + h_2/h_3\ h_4)$	0.09	0.10
CV	9%	17%

[a] μ = mean; σ = standard deviation; h_i = intensity maximum i; i = 1, 2, 3, 4; CV = (σ/μ)* 100 %. All values except CV are given in arbitrary units.

erature a really fast, clear labelling by PRINS (about 30 min) can only be achieved if about 5-10 µg DNA probe were used. This number may be compared to about 70 *ng* required in our approach. For routine clinical application this should be also taken into consideration.

It is well-established knowledge that the stringency condition of a FISH protocol is dependent on the percentage of formamide used and thus the minor binding

sites of repetitive DNA probes can be suppressed. The results presented here (showing an increased number of minor binding sites with increasing hybridization time) are compatible with this theory. Nevertheless, for the DNA probes used here, the fluorescence intensities of the major binding sites differed significantly from the other hybridization regions if one chooses the appropriate hybridization time (compare, for instance, Fig. 7 and Table 3). This agrees well with results known from radioactive labelling with the examined DNA probes (27, 29). In routine applications, such clearly distinguishable fluorescence intensities may allow to evaluate the images by simple procedures of digital image analysis (13, 49, 54). This can be done with a normal PC connected to a CCD image detection system. During the last few years such systems became more and more economical and easy to handle so that nowadays this technique does not appear to be essentially more complicated than microscope photo cameras.

In this paper, two repetitive DNA probes were hybridized to human metaphase chromosomes and interphase nuclei of human peripheral lymphocytes. Hybridization of these types of probes should result in two hybridization spots corresponding to the two major binding sites on the homologous chromosomes, towhich the DNA probes are known to be specific. If it is expected that each chromosome contains the same amount of target DNA and if it is expected that DNA-DNA in situ hybridization is quantitative, the hybridization signals should have the same fluorescence intensity on the homologous spots. Recently published results (48) obtained from FISH with alphoid probes forchromosome 1 and 7 using a well-established formamide FISH procedure with different detection systems
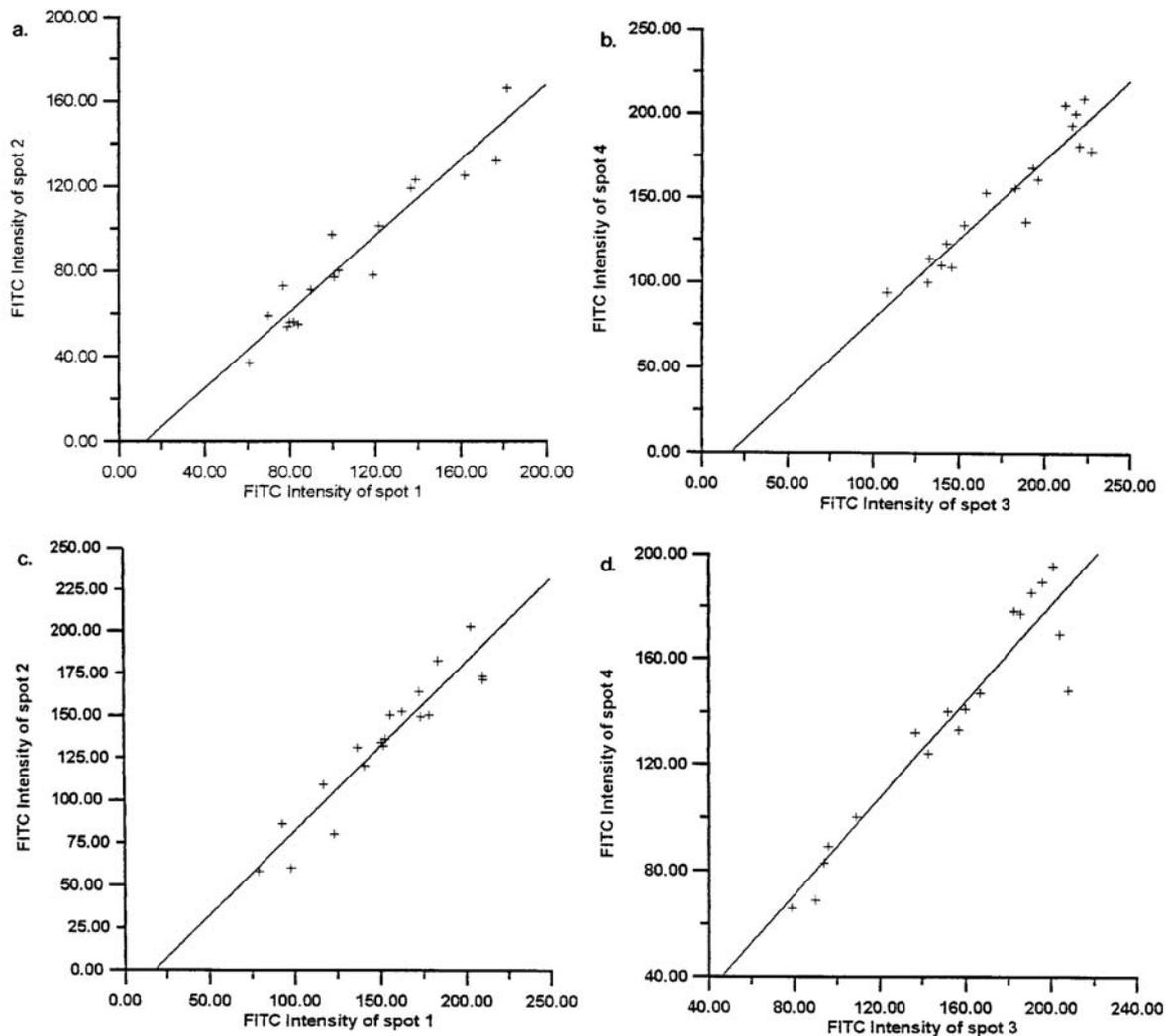
21

FIG. 7 Intensities for spots in metaphase spreads hybridized with the D15Z1 probe, (a) h_2 vs. h_1, t_n = 30 mm (presumptive chromosome 15); (b) h_4 vs. h_3, t_n = 30 min (presumptive chromosome 9); (c) h_2 vs. h_1, t_n = 15 min (presumptive chromosome 15); (d) h_4 vs. h_3, t_n = 15 min (presumptive chromosome 9)'

showed that there exists a large inter-nuclear as well as intra-nuclear variation in the fluorescence intensity of homologous spots. While the inter-nuclear variations may be explainable by microscopical and cyto-chemical reasons, these intra-nuclear variations were in contrast to the above-mentioned assumptions. One reason might be a differential DNA loss in the target DNA during the preparative steps in the hybridization procedure.

So far, the results presented here in general are well compatible to the results of (48) with respect to intracellular fluorescence variation. Moreover, comparing the spot 1 to spot 2 correlations, the CV observed here (Table 2) might be even somewhat smaller. Compared

to Nederlofet al. (48), the technique presented here has a lower number of preparative steps and avoids formamide or other equivalent chemical denaturing agents. This might further reduce possible DNA loss. The *reduced* CV observed for metaphase spreads compared to interphase nuclei might support this argument.

The limited depth of field of the microscope objective used is often said to be a problem. Its high numerical aperture collects as much light as possible and it allows a high spatial resolution for the applied image analysis device. The use of such an objective appeared to be necessary in order to facilitate the detection of less fluorescent minor binding sites of the applied DNA probes and to discriminate the different localization of the

Table 4
Average Size (±S.D.) of Spot Area in Pixel Numbers[a]

	30 sec	15 min	30 min
pUC 1.77 nuclei			
Chromosome 1	112.9 ± 44.1	125.5 ± 56.6	118.2 ± 60.0
Chromosome 9, 15, or Y	65.0 ± 36.5	70.3 ± 43.3	72.9 ± 34.7
pUC 1.77 metaphase spreads			
Chromosome 1	127.0 ± 52.6	132.1 ± 49.2	139.5 ± 55.4
Chromosome 9, 15 or Y	54.8 ± 31.6	73.6 ± 40.4	79.1 ± 39.9
D15Z1 metaphase spreads			
Chromosome 15		94.1 ± 29.6	90.6 ± 33.4
Chromosome 9		142.9 ± 43.3	151.1 ± 45.8

[a]The chromosome identifications are presumptive and depend on the assignment according to the relative chromosome length and centromere position. For number of cells evaluated, see Tables 2 and 3.

FISH signals. From theory, the calculated depth of field should be sufficient in the case of metaphase spreads. In the case of nuclei, however, the depth of field should be in the order of the depth of cell nuclei (more than 2 μm). Here, the technical reason may be found for the higher CV compared to the corresponding CV of the metaphase spreads. But the sharpness of the images registered without any edge enhancing operation reduces the importance of this argument. Moreover, a lesser depth of focus may also contribute to the CV of the FISH signals in the nuclei so that the real intra-nuclear CV due to DNA loss may be even lower. The problem of depth of focus for the detection of FISH signals in cell nuclei can be overcome if the nuclei can be rotated in a capillary, like in a micro axialtomographical device (5, 67). Choosing an appropriate rotation angle would bring the homologous FISH signals into the same focal plain.

We have shown a fast alternative non-radioactive in situ hybridization protocol without the use of enzymes, formamide, or other denaturing chemical agents. Up to now, the technique has only been described for two repetitive probes (pUC 1.77, D15Z1). Even so, advantages for routine investigations are apparent. However, for general screening purposes, especially in tumor diagnostics, other repetitive probes, and single copy probes, have to be included. This opens a broad field for further examinations to optimize hybridization conditions and probes for short time protocols, in combination with digital image analysis. It is anticipated that the problem of the occurrence of minor binding sites may be resolved by using appropriate cocktails of single copy probes or highly specific oligonucleotides. The effort required to establish such probe cocktails for special routine applications should be compensated for by the possibility of a rapid FISH procedure. Even FISH applications to tissue analysis during a surgical intervention might eventually become possible.

The technique may also be attractive for FISH in suspension not only for cell nuclei (62) but also for metaphase chromosomes (21). Together with new de-

velopments in slit-scan flow cytometry (for review see, for instance, 11) this might be a technique for fast aberration screening (28) of large numbers of chromosomes in a relatively short time.

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