Research Reports

A Rapid FISH Technique for Quantitative Microscopy

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ABSTRACT

Results of quantitative microscopy for fluorescence in situ *hybridization (FISH) signals with repetitive DNA probes (pUC 1.77 and D15Z1) are reported. A nonenzymatic hybridization technique was applied using fluorescein-12-dUTP labeled DNA probes and a buffer system not containing any formamide or equivalent chemical denaturing agents. Following thermal denaturation, the renaturation time was re duced to less than 30 min. The number of wash steps was reduced to one. For the pUC 1.77 probe, the major binding sites (chromosome 1) were distinguished from the minor binding sites by means of fluorescence intensity and spot size. The intensity variation of the two brightest FISH spots (major binding sites) in the same metaphase was 19% for 15 min renaturation time and 16% for 30 min renaturation time. For the D15Z1 probe, generally four bright spots were visible and tentatively assigned according to chromosome length and centromere position (chromosomes 15 and 9). The intensity variation of each two homologues in the same metaphase spread showed a coefficient of variation of 47% (15 min) and 22% (30 min) for chromosome 15, and 19% (15 min) and 15% (30 min) for chromosome 9. The results indicate that the applied technique can considerably accelerate the FISH procedure and is suited for quantitative microscopy.*

INTRODUCTION

Fluorescence *in situ* hybridization (FISH) allows the detection of specific DNA and RNA sequences at the individual cell level. It has become an important tool for cell biology, chromosome research and cytogenetic diagnostics (7,16,17,23,24). During the last few years the sensitivity of FISH has improved considerably. All over the world cytogeneticists have established *in situ* hybridization protocols that have been routinely used in their laboratories. More and more, these protocols have become important for clinical applications (23). However, in the case of clinical routine, it would be desirable to accelerate and to automate the hybridization process.

A useful step in realizing these demands was the introduction of fully automatized polymerase chain reaction (PCR) techniques (18) for probe amplification for FISH (15, 25). This was also done in combination with the direct incorporation of labeled nucleotides (5, 26) so that nick translation became unnecessary. An interesting alternative to classical FISH has been introduced called PRINS (primed *in situ* labeling) (13,14). Compared to FISH, PRINS eliminates the need for separate probe labeling. PRINS was considerably faster than classical FISH for the same test DNA probes but required higher probe concentrations(10).

Recently, a new FISH procedure has been developed (3) that results in renaturation times compatible to the PRINS technique. This FISH protocol has been based on the principle of avoiding denaturing chemical agents, such as formamide in the hybridization buffer, so that a number of time-consuming wash steps have become unnecessary (4). Thus, the most time-consuming step of the entire procedure has become the immunochemical probe staining after *in situ* hybridization. Here, a further development of this new FISH protocol is presented that applies fluorescein isothiocyanate (FITC)-labeled nucleotides in probe preparation. Although FITC-labeled DNA probes are known to show less intense signals for direct visualization (1), the results presented here indicate that they are all well suited for interactive

image analysis by quantitative microscopy.

MATERIALS AND METHODS

Slide Preparation

Metaphase chromosomes and cell nuclei were obtained from human lymphocytes isolated from peripheral blood by standard techniques (2). The lymphocytes were stimulated by Phytohemagglutinin M (2.5 µg/mL chromosome medium; Bi Seromed, Biocnrom KG, Berlin, FRG) and cultivated for 72 h followed by a Colcemid® block (27 uM) (Boehringer Mannheim, Mannheim, FRG) for 2 h. The cells were treated according to a modified hexandiol method (9), and the metaphase spreads and interphase nuclei were fixed on slides by means of methanol/acetic acid (3:1, vol/vol) (21). No further treatment (such as dehydration, proteinase digestion, etc.) was used to prepare the slides for FISH.

Preparation of DNA Probes

For the pUC 1.77 DNA probe (6) and for the D15Z1 DNA probe (22), entire plasmids with the human insert (kindly provided by Dr. T. Cremer, Institute of Human Genetics and Anthropology, Heidelberg) were labelled with fluorescein-12-2'-desoxy-uridine-5-triphosphate (fluorescein-12-dUTP) (Boehringer Mannheim) by nick translation (Nick Translation Kit; Boehringer Mannheim) according to the product information with the following modifications: (i) Five units DNA polymerase I (Boehringer Mannheim) were additionally added to the reaction volume. (ii) The absolute amount of fluorescein-12-dUTP was raised to 4 nmol.

The nick translation step may be replaced by PCR incorporation of FITC-labeled nucleotides (5, 15, 18, 26).

In Situ Hybridization and Detection

In situ hybridization without formamide or equivalent chemical denaturing agents was performed as described in detail elsewhere (4). Briefly: Approximately 70 ng of the labelled DNA probe, 3 µL hybridization buffer [l0x: 100 mmol/L Tris-HCl, 30 mmolTL $MgCl_2$, 500 mmol/L KCl, 100 Hg/mL gelatin, pH 8.3 (20°C)] and 3 µL 20x standard saline citrate (SSC) were diluted in deionized H_2O to make up a final volume of 30 µL. This hybridization mixture was pipetted on the microscope slides with the fixed metaphase spreads. The slides were covered with a cover glass, sealed with rubber cement (Fixogum; Marabu, Tamm, FRG) 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 (D. Adam, M. Hausmann, C. Cremer, unpublished results).

For renaturation, the steel chamber with the slides was placed into a water bath at 40°C for 30 min, or 15 min, respectively. For detection, the slides were washed once in a solution of 0.9% NaCl/0.2% Tween® 20 for 5 min at room temperature and then air-dried. For counterstaining of the chromosomes, propidium iodide (PI) (5 µmol/L or 15 µmol/L, respectively) or diamidinophenylindole (DAPI) (5 µmol/L) was used.

Microscopy and Digital Image Analysis

For visualization, a fluorescence microscope (Leitz Orthoplan; Leica, Wetzlar, FRG) was used equipped with a Plan-APO 63x/NA 1.40 objective and a 50-W mercury arc lamp. Excitation took place by use of a band pass filter (450-490 nm) and detection by use of a 515-nm-long pass filter. On the slides, metaphase spreads were chosen by random access and their fluorescence images were registered by a cooled color charge-coupled device (CCD) camera (CF 15 MC; Kappa, Gleichen, FRG) having an interline CCD chip with integrated mosaic filter and a resolution of 460 x 440 pixels. The images were transferred to a color frame grabber (ITI Vision Plus Color CFG 512; Kappa). For registration and interactive evaluation, the commercially available software package OPTIMAS (BioScan, Edmonds, WA, USA) was running on a PC (80486) under WINDOWS® 3.1 with the MS-DOS® operating system. This software package allows the direct implementation of program subroutines designed for the special purpose of the evaluations presented here.

For all images, a constant acquisition time *of* 9 s was chosen to allow a direct comparison with previous results obtained by antibody visualization of digoxigenin-labeled probes (3). This also reduces systematic errors due to illumination effects or photo bleaching of FITC (H. Tanke, Leiden, personal communication). The digitized original images were visualized and evaluated without any further processing such as filtering, contrast-enhancing operations or background corrections. All quantitative results were obtained from the green image plane ("green light" contribution to the real color image) using a sequence *of* OPTIMAS subroutines. To register integrated intensity profiles, a line was interactively fixed along the chromosome axis of those four chromosomes with the brightest spots. The width of the line was chosen such that the width of a spot was entirely covered. For background subtraction, a subroutine was written that automatically determines the bor-

Table 1. Evaluation of the Hybridization Spots on Metaphase Spreads after FISH with pUC 1.77 for Different Renaturation Times

	15 min	30 min
Number of metaphase spreads	21	20
Mean $\mu(h_1)$	54.57	63.6
Mean $\mu(h_2)$	39.57	46.45
Standard deviation $\sigma(h_1)$	13.46	14.36
Standard deviation $\sigma(h_2)$	10.98	11.95
$\mu(h_1/h_2)$	1.41	1.40
$\sigma(h_1/h_2)$	0.27	0.23
Coefficient of variation (CV)	19%	16%
$\mu(h_3/h_4)$	1.24	1.20
$\sigma(h_3/h_4)$	0.22	0.12
CV	18%	10%
$\mu(h_1 + h_2)$	94.14	110.05
$\sigma(h_1 + h_2)$	23.20	25.10
CV	25%	23%
$\mu(h_3 + h_4)$	75.92	55.15
$\sigma(h_3 + h_4)$	23.31	20.80
CV	31%	38%
$\mu\left(\frac{h_1 + h_2}{h_3 + h_4}\right)$	1.41	1.95
$\sigma\left(\frac{h_1 + h_2}{h_3 + h_4}\right)$	0.35	0.45
CV	25%	23%

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.

der on both sides of the labeling peak using the first derivative *of* the profile. The intensity below the direct connection *of* these minima was defined as background (an interactive control of this procedure showed only differences *of* a few percent). Then the remaining intensity maxima h_i (i = 1,...,4) were determined in arbitrary units (a.u.). In the following, these integrated intensity maxima will be referred to as "intensities." To characterize the hybridization quality and to offer quantitative parameters for comparison to other hybridization techniques (see, e.g., Reference 19), the intensities of the chromosomes with major *(h_1, h_2)* and minor *(h_3, h_4)* binding sites were studied in detail. For this purpose h_1/h_2 and h_3/h_4 (intensity variation on chromosomes), $h_1 + h_2$ and $h_3 + h_4$, as well as $(h_1 + h_2)/(h_3 + h_4)$ (comparison *of* major and minor binding sites), were determined. The mean value μ, the standard deviation σ and the coefficient of variation (CV) were calculated using the formula CV = 100% x σ/μ. In addition, the relative areas *of* hybridization spots were determined in numbers of pixels. Fluorescence signals were evaluated only if they clearly exceeded the background level (about 10 a.u.).

Table 2. Average Number (:tSD) of Minor Binding Sites Per Metaphase Spread with a Signal Intensity Higher Than Background Fluctuations

	15 min	30 min
pUC 1.77	1.76 ± 0.79 (n = 21)	2.05 ± 0.55 (n = 20)
D15Z1	4.22 ± 2.9 (n = 18)	9.05 ± 3.3 (n = 19)

All hybridization spots above background were counted except the signals from the presumptive chromosome 1 (pUC 1.77 probe) and from the presumptive chromosome 15 (D15Z1 probe), respectively. (n = number of evaluated metaphases)

RESULTS

The influence *of* two different renaturation times t_n on the fluorescence signals *of* major and minor binding sites *of* two repetitive probes (pUC 1.77, D15Zl) with well-known chromosomal 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 distinctly visible for both probes. Although the intensity *of* the hybridization spots in general appeared to be lower than for digoxigenin-labeled probes using the same hybridization technique (3), the signals were clearly visible by microscopic inspection and well suited for computer-aided quantification by digital image analysis. The hybridization sites were compatible with the locations of the probes as described in literature (11, 12). Fluorescence signal noise due to FITC-background was below the sensitivity of the detection system.

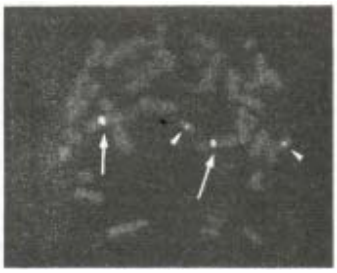

Figure 1. Microphotograph of a human lymphocyte metaphase spread after FISH with the pUC 1.77 probe (t_n = 15 min). Arrows: major binding sites on chromosome 1 corresponding to signal intensities h_1, h_2; arrowheads: minor binding sites corresponding to signal intensities h_3, h_4.

Figure 1 shows the microphotograph of a typical example for the pUC 1.77 probe after FISH with a renaturation time *of* t_n = 15 min. On all metaphases examined for t_n =15 min and t_n =30 min, the two major binding sites were localized on the longest chromosomes compatible with the location on chromosome 1 (11). Hybridization with the pUC 1.77 probe resulted in a low number of minor binding sites (between 0 and 4) on medium-sized or small chromosomes. According to the

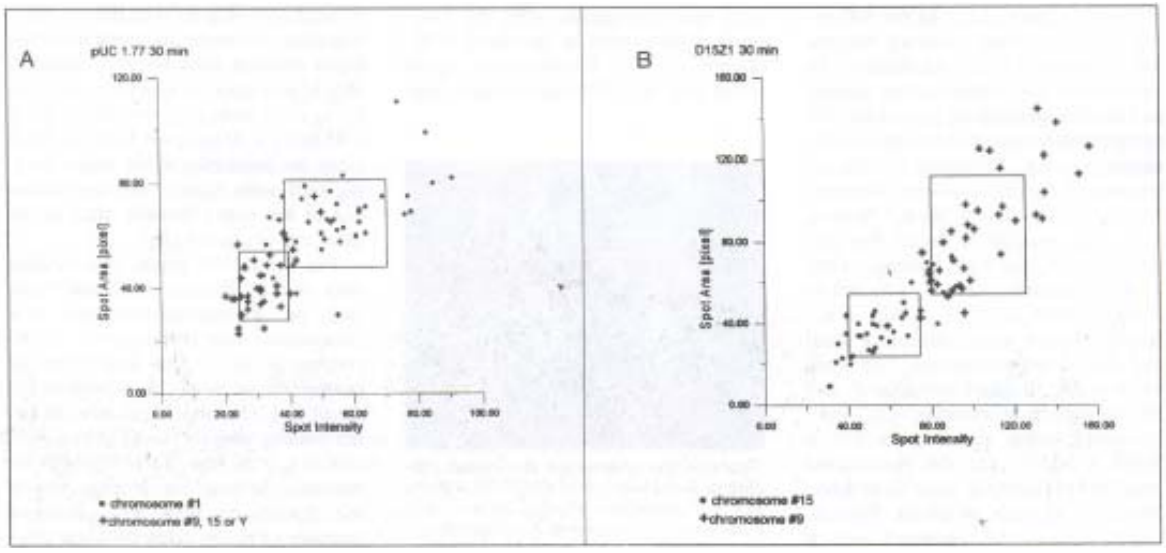

Figure 2. Spot areas as a function of spot intensities for t_n= 30 min. A) pUC 1.77 probe (dots represent the major binding site on chromosome 1; crosses represent the minor binding sites on chromosomes 9,15 and Y). B) D15Z1 probe (dots represent the major binding site on chromosome 15;crosses represent the minor binding site on chromosome 9). Borderlines of the areas in the plots are determined by the σ distance around the mean value. (For further details see text.)

relative chromosome length and the centromere position, these locations were compatible with locations on chromosomes 9, 16 or Y (11). The number of those minor binding sites increased only slightly with the renaturation time. However, the contrast of the major binding sites became considerably higher *[μ(h_1 + h_2/h + h_4)*=1.41 for t_n = 15 min; *μ(h_1 + h_2/h_3 + h_4)* = 1.95 for t_n =30 min; see Table 1]. In all cases the intensities of the major binding sites were higher than the intensities of the minor binding sites in the same cell *(h_3, h_4 < h_1, h_2).*

For the D15Z1 probe, two binding sites of high fluorescence were localized on a chromosome that was compatible with chromosome 15 according to the relative length and the centromere position (12). Compared to the *pUC* 1.77 probe, the number of minor binding sites increased from t_n =15 min to t_n =30 min (Table 2). With the renaturation time, the average size of the fluorescent area, also increased (number of pixels using the same magnification in all experiments) for both probes (Table 3). Especially for the D15Z1probe, the size of the spots on a chromosome compatible with chromosome 9 (according to the relative length and centromere position), which is known as a minor binding site of this probe (12), increased even if the relation μ*(h_1* + h_2/h_3 + h_4) did not change. By visual inspection, these spots appeared to be much brighter than the values and relations of the calculated intensities expressed (Table 4). The other minor binding sites of the D15Z1 probe never showed similar intensities so they were not investigated further.

Figure 2 shows the spot areas (in pixels) as a function of the spot intensities (in a.u.) for the pUC 1.77 probe (Figure 2A) and the D15Z1 probe (Figure 2B).

Table 3. Average Size (±SD) of SpotArea in Pixel Numbers

	15 min	30 min
pUC 1.77		
Chromosome 1	53.8 ± 20.2	66.0 ± 14.8
Chromosome 9, 15 or Y	31.3 ± 9.0	43.1 ± 12.4
D15Z1		
Chromosome 15	38.3 ± 17.8	40.1 ± 14.9
Chromosome 9	47.1 ± 20.5	64.2 ± 27.7

The chromosome identifications are presumptive and depend on the assignment according to the relative chromosome length and centromere position.

Mean values of intensity and spot size were calculated *for* the two types of spots (shown by dots and crosses). For Figure 2A, μ_1 represents the mean for the minor binding sites (crosses); for Figure 2B, μ_1 represents the mean *for* the major binding sites (dots). The means μ_2 were calculated for the major binding sites in Figure 2A (chromosome 1; dots) and the minor binding sites (chromosome 9; crosses) in Figure 2B. The higher intensity of the minor binding site (chromosome 9) in the case of D15Z1 is probably due to low-stringency conditions of the applied FISH technique (12) Around the mean values μ_1 and μ_2, an area was calculated with the border lines at a distance of $\pm\sigma_{1,2}$ (assuming a 2-dimensional Gaussian distribution). Misclassification within these areas was between 10% and 25%. Most of the spots of one type of FISH sites that were found outside these areas were either at values lower than $\mu_1 - \sigma_1$ or at values higher than $\mu_2 + \sigma_2$. In these cases no "false" events were found. This means no spots of type 1 were found at positions ≥ $\mu_2 + \sigma_2$ and no spots of type

2 at positions ≤ $\mu_1 - \sigma_1$. For example, in Figure 2A all spots with an area and an intensity lower than $\mu_1 - \sigma_1$ represented minor binding sites, and all spots with an area and an intensity higher than $\mu_2 + \sigma_2$.represented major binding sites. These results indicate that major and minor binding sites can statistically be discriminated by digital image analysis using the parameters "spot area" and "spot intensity."

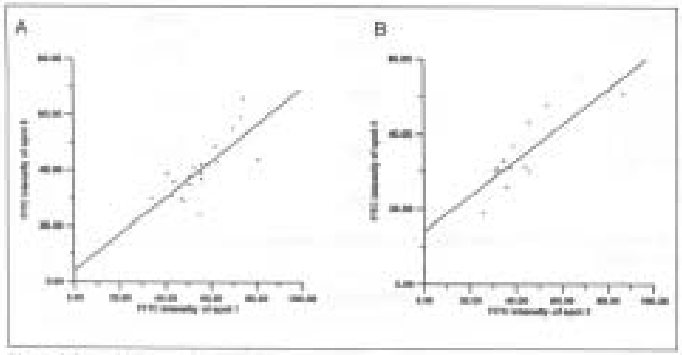

Figure 3. Intensities h_2 vs. h_1 (A) and intensities h_4 vs. h_3 (B) for t_n = 15 min in a.u. for metaphase spreads hybridized with tbe pUC 1.77 probe.

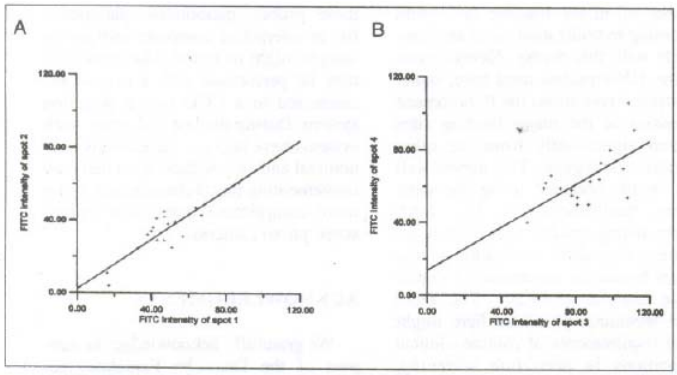

Figure 4. Intensities h_2 vs. h_1 (A) and intensities h_4 vs. h_3 (B) for t_n = 15 min in a.u. for metaphase spreads hybridized with the D1SZ1 probe.

Figure 3 shows the variation of the spot intensities on the metaphase chromosomes after hybridization with the pUC 1.77 probe for the renaturation time t_n = 15 min. 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)* (two homologue chromosomes 1; Figure 3A). For the third *(h_3)* and fourth highest intensity *(h_4),* an analogous representation was calculated (Figure 3B). By means of linear regression analysis, a regression line was determined. Similar results were obtained for t_n =30 min (data not shown). In Table 1 the results for the pUC 1.77 probe are summarized. The means $\mu(h_1/h_2)$ (intracellular variation) were nearly the same for both t_n and showed similar CVs (19% and 16%). The intercellular variation [CV of $\mu(h_1 +.,h_2)$]was also very similar for t_n=15 min and for t_n = 30 min (25% and 23%, respectively). In the case of the two minor binding sites, the CVs of $\mu(h3/h4)$ were 18%mand 10%. The CVs of $\mu(h3+ h4)$ were 31% and 38%. A clear correlation to the renaturation time was not apparent for the pUC 1.77 probe. Only the contrast between the major and the minor binding sites, which is expressed by $\mu(h_1 + h_2/h_3 + h_4)$, slightly increased with t_n.

Figure 4 shows examples for the variation of the spot intensities for the D15Z1 probe. The graph h_2 as a function of h_1 represents the signal intensities for chromosome 15 (Figure 4A) and h_4 as a function of h_3 represents the signal intensities for chromosome 9 (Figure 4B). By means of linear regression analysis, a regression line was determined. Similar results were obtained for t_n = 30 min (data not shown). In Table 4 the results for the D15Z1 probe are summarized. With increasing t_n the means $\mu(h_1/h_2)$ and $\mu(h_3/h_4)$ tended towards 1 and the corresponding CVs decreased. The intercellular variation for chromosome 15 [CV of $\mu(h_1 + h_2)$] decreased considerably with t_n (from 36% to 27%), while it nearly was constant for chromosome 9 [CV of $\mu(h_3+ h_4)$]. The relation $\mu(h_1 + h_2/h_3+ h_4)$ was nearly constant for both renaturation times, but the CV of 19% was much better for t_n =30 min than for t_n = 15 min, which showed a CV of 35%.

DISCUSSION

To simplify the FISH procedure for specific labeling of chromosome regions with repetitive DNA probes, we have previously described a nonenzymatic technique working without any formamide or equivalent denaturing chemical agents (except methanol: acetic acid fixation) (4). Simultaneously, the slide preparation was further simplified (no ethanol dehydration, proteinase digestion, etc.). It was also possible to reduce the renaturation time required for reasonable fluorescence hybridization to less than 30 min. Due to the immunofluorescence step (ca. 2 h), however, the total FISH procedure still took a couple of hours (3).

In the report presented here, FITC labeled nucleotides were directly incorporated into the DNA probes. Thus, the incubation time required for the detection by immunofluorescence of the hybridized DNA probes was omitted. In this way, a further, substantial reduction of the total amount of time necessary to perform the entire FISH procedure was achieved. As another consequence, just one wash step remained necessary for the entire FISH protocol, which can, in principle, now be performed in a minimum of approximately half an hour or less.

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 used. This opens a broad field for future investigations into optimizing hy-

bridization 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. The effort required to establish such probe cocktails for special routine applications should be highly compensated by the possibility of a rapid FISH procedure. Even FISH applications to tissue analysis during a surgical intervention might eventually become possible.

Compared to the PRINS technique (13,14), the FISH procedure described here can be performed in about the same time using similar technical equipment. It may be pointed out, however, that according to the literature, a really fast, clear labeling 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 also be taken into consideration.

It is a well-established fact that the stringency condition of a FISH protocol is dependent on the percentage of formamide or other chemical denaturing agents 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, under appropriate conditions the fluorescence intensities of the major binding sites differed significantly from the other hybridization regions. This agrees well with results obtained using the same probes, radiolabeled (11, 12). Such clearly distinguishable fluorescence intensities may allow evaluation of the images by simple procedures of digital image analysis (8, 19, 20). The rapid FISH technique presented here might fulfill requirements of routine clinical applications in aberration screening, where a standard set of hybridization probes is often used (e.g. for the detection of numerical aberrations). For those probes, quantitative parameters for an interactive computer analysis of images might be found. The evaluation may be performed with a normal PC connected to a CCD image detection system. During the last few years, such systems have become increasingly economical and easy to handle, so that now implementing this technique may be no more complicated than using microscope photo cameras.

ACKNOWLEDGMENTS

We gratefully ackn6wledge the support of the Deutsche Forschungsgemeinschaft (Cr 60/7-2). We also thank Dr. med Durm, Frankenthai, for providing the lymphocytes and Dr. T. Cremer for stimulating discussions.

Table 4. Evaluation of the Hybridization Spots on Metaphase Spreads After FISH with D15Z1 for Two Different Renaturation Times

	15 min	30 min
Number of metaphase spreads	18	19
Mean $\mu(h_1)$	46.67	64.37
Mean $\mu(h_2)$	34.00	52.05
Standard deviation $\sigma(h_1)$	16.62	18.84
Standard deviation $\sigma(h_2)$	13.09	15.04
$\mu(h_1/h_2)$	1.54	1.26
$\sigma(h_1/h_2)$	0.73	0.28
Coefficient of variation (CV)	47%	22%
$\mu(h_3/h_4)$	1.36	1.14
$\sigma(h_3/h_4)$	0.26	0.17
CV	19%	15%
$\mu(h_1 + h_2)$	80.67	116.42
$\sigma(h_1 + h_2)$	28.69	31.89
CV	36%	27%
$\mu(h_3 + h_4)$	149.33	206.79
$\sigma(h_3 + h_4)$	30.11	42.90
CV	20%	21%
$\mu\left(\frac{h_1 + h_2}{h_3 + h_4}\right)$	0.55	0.56
$\sigma\left(\frac{h_1 + h_2}{h_3 + h_4}\right)$	0.20	0.11
CV	35%	19%

μ = mean; σ = standard deviation; h_i = intensity maximum i; i = 1, 2, 3, 4; CV = $(\sigma/\mu) \times 100\%$.

All values except CV are given in arbitrary units.

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Received 16 August 1993; accepted 1 April 1994.

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