Optimization of Fast-Fluorescence *in situ* Hybridization with Repetitive α-Satellite Probes

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A rapid FISH (fluorescence *in situ* hybridization) technique (Fast-FISH) for quantitative microscopy has been recently introduced. For highly repetitive DNA probes the hybridization (renaturation) time and the number of necessary washing steps were reduced considerably by omitting formamide or equivalent denaturing chemical agents. Due to low stringency conditions major and minor binding sites of the probes used showed visible FISH signals well suited for quantitative image-microscopy. The discrimination of minor and major binding sites was possible by automated image-processing. Here, a further, quantitative optimization of the Fast-FISH technique is described that allows to clearly discriminate major and minor binding sites of α-satellite probes by an easy image classification parameter. With respect to the optimization it was necessary to verify two sensitive parameters (hybridization time and temperature) of the given rapid FISH protocol. As examples the systematic optimization for the two probes D12Z2 (major binding site on the centromere of chromosome 12) and D8Z2 (major binding site on the centromere of chromosome 8) are shown. The optimal hybridization conditions concerning rapidness and quality of chromosome morphology were obtained using a hybridization temperature of 70 °C and a hybridization time of 60 min. For these conditions major and minor binding sites were clearly discriminated by the intensity maximum S_{max} of the corresponding FISH-spots.

Introduction

Fluorescence *in situ* hybridization (FISH) allows the individual delineation of chromosomes and chromosomal subregions (for review see Cremer and Cremer, 1992; Gray *et al.*, 1994; Lichter *et al.*, 1991; Trask, 1991). Repetitive centromere probes as for instance α-satellite probes are routinely used in chromosome research, clinical diagnostics, or radiation biology. Meanwhile, a broad spectrum of those probes in combination with hybridization reaction kits is commercially available. These reaction kits as well as most of the established FISH protocols (Lichter and Cremer, 1992) are based on developments from earlier *in situ* hybridization procedures (Langer-Safer *et al.*, 1982; Pinkel *et al.*, 1986; Schardin *et al.*, 1985). In these protocols usually denaturing organic chemical agents are applied on both, the DNA probe and the chromosomal target. Especially formamide in high concentration with moderate heat denaturation has been established as a standard essential ("formamide protocol"). This treatment is referred to allow high stringency conditions, i. e. minor binding sites of DNA probes can be suppressed so that ideally only one (= the specific) binding site remains labeled. However, consequences of these advantages using formamide are a long hybridization time (typically overnight up to several days (e. g. Tucker *et al.*, 1993)) and a complex, workloaded washing procedure afterwards. In the following, hybridization time and hy-

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bridization temperature mean the time and temperature required for probe-target DNA renaturation only.

In the case of clinical routine diagnostics (Carter, 1994) or biological dosimetry (Cremer *et al.*, 1995; Cremer *et al.*, 1990; Gray *et al.*, 1994) it is highly desirable to accelerate the entire hybridization procedure and to develop easy to handle, well reproducible protocols. It has been observed (Celeda *et al.*, 1992) that FISH of repetitive DNA probes is feasible also in the absence of formamide or equivalent denaturing agents. Starting from these findings, a modified FISH technique ("non-formamide protocol") with a high temperature denaturation treatment has been described (Celeda *et al.*, 1994; Haar *et al.*, 1994). For the two highly repetitive probes pUC 1.77 (specific for the region 1q12) and D15Z1 (specific for centromere of chromosome 15), it was possible to shorten the hybridization time considerably down to a minimum of 30 sec (Fast-FISH). Incorporation of fluorescein labeled nucleotides into the DNA probes reduced the number of subsequent washing steps to one so that the complete FISH procedure was finished in less than half an hour. Due to the lower stringency conditions, minor binding sites became also visible. However, for pUC 1.77 and D15Z1, major and minor binding sites were discriminated by computer image analysis. For this purpose a classification algorithm on the basis of spot intensity and spot area evaluation was written (Aldinger *et al.*, manuscript in preparation). Although this approach lead to reasonable results in many cases, it was desirable to further study the Fast-FISH procedure and to optimize the hybridization parameters. In the "non-formamide protocol", formamide and other organic chemical denaturing agents have been eliminated. Therefore, hybridization time and hybridization temperature acquire a still more prominent role as two highly sensitive basic parameters for the hybridization process. Additional influence of still unknown significance might be for example, the state of condensation and "aging" of the chromosomal targets, the consistance and the pH of the buffer, and the type of chemical modifications used to label the DNA probes. Here, we will show by quantitative image microscopy, how the two major parameters, hybridization time and temperature influence the hybridization behaviour of α-satellite probes, and how this effect can be used to easily discriminate major and minor binding sites.

Materials and Methods

Slide preparation

Metaphase chromosomes were obtained from human lymphocytes isolated from peripheral blood by standard techniques (Boeyum, 1964). The lymphocytes were stimulated by phytohemagglutinin M (2.5 µg/ml lymphocyte medium) and cultivated for 72 hours followed by a Colcemid block (Boehringer Mannheim, Mannheim, FRG) for the last two hours. The treatment of the cells was according to a modified hexandiol method (Dudin *et al.*, 1987), and the metaphase spreads and interphase nuclei were fixed on slides by means of methanol/acetic acid (3:1, v/v). The slides were used for FISH without further treatment (such as dehydration, different kinds of digestion etc.).

Preparation of DNA probes

For the chromosome 12 α-satellite probe D12Z2, the entire plasmid with the human insert (kindly provided by Prof. Dr. T. Cremer, Institute of Human Genetics and Anthropology, University of Heidelberg) was labeled with digoxigenin-11-dUTP by nick translation (Nick Translation Kit, Boehringer Mannheim, FRG) according to product information. The chromosome 8 α-satellite probe D8Z2 was a digoxigenin labeled commercial probe from ONCOR, Inc. (ONCOR, 209 Perry Parkway, Gaithersburg, MD 20877).

In situ hybridization

In situ hybridization without formamide or equivalent chemical denaturing agents (referred to as "without formamide" or "non-formamide protocol") was performed as described in detail elsewhere (Celeda *et al.*, 1994).

Briefly: Approx. 50 ng of the labeled DNA probe, 3 µl hybridization buffer (10×: Tris[2-amino-2-(hydroxymethyl)-1,3-propanediol]-HCl 100 mmol/l; $MgCl_2$ 30 mmol/l; KCl 500 mmol/l; gelatine 10 mg/l; pH 8.3 (20 °C)), and 3 µl 20×SSC (final pH around 8) 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 for denaturation and hybridization.

Simultaneous thermal denaturation of probes and chromosomal targets was performed at 95 °C for 5 min. This denaturation temperature was estimated from hyperchromicity curves registered at a wavelength of about 260 nm for isolated human lymphocyte metaphase chromosomes and different probes in the hybridization buffer used (D. Adam, J. Rauch, D. Wolf, M. Hausmann, C. Cremer, unpublished results). For hybridization the steel chamber with the slides was placed into a waterbath of a: 40 °C for (15, 30, 60, and 120 min); b: 60 °C for (15, 30, 60, and 120 min); c: 70 °C for (15, 30, 60, and 120 min); d: 75 °C for (15, 30, 60, and 120 min).

For detection the slides were washed once for 5 min at RT in a solution of 0.9% NaCl/0.2% Tween 20. For fluorescence labeling with antidigoxigeninfluorescein Fab fragments (Boehringer/ Mannheim GmbH, Mannheim, FRG) the slides were incubated for 2 h at 37 °C and washed afterwards again in 0.9% NaCl/0.2% Tween 20. For counterstaining of the chromosomes, propidium iodide (PI) (0.2 μg/ml) and diamidinophenylindole (DAPI) (5 μmol/l) were used.

Digital image analysis

For visualization, an image analysis system described in detail elsewhere (Aldinger *et al.*, manuscript in preparation; Bornfleth *et al.*, 1996) was supplied. It is based on a fluorescence microscope (Leitz Orthoplan, Leica, Wetzlar, FRG) equipped with a Plan APO 63×/NA 1.40 objective and a 50 W mercury arc lamp. Excitation took place *via* a 450–490 nm band pass filter and emission *via* a 515 nm long pass filter. On the slides, metaphase spreads were chosen by random access. For each hybridization temperature and each hybridization time, 30 metaphase spreads were recorded using a cooled color CCD-camera (CF 15 MC, Kappa, Gleichen, FRG). A constant acquisition time for each slide was chosen. The images were transferred to a color frame grabber (ITI Vision Plus Color CFG 512; Kappa). For registration and 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. In this software package a program subroutine was implemented, which was designed for automated spot finding and evaluation. From the green image plane of the RGB (Red, Green, Blue)-image all quantitative data of the FISH spots were obtained. The program automatically analyzed all regions of high intensity. The spot areas were segmented by calculating the intensity distribution around the maximum intensity. At the points comparable to local maxima in the second derivative around the intensity maximum, the borderline of the spot was fixed. For every FISH-spot in a metaphase spread, S_{max} (maximum intensity) and A (area) were computed. In addition the normalized intensity values (normalized to the intensity of the brightest FISH spot in each metaphase spread) were calculated. For each experiment 30 randomly chosen metaphases were averaged and the mean values and standard deviations were computed for the other spots. All these data were further processed by a standard spreadsheet program and visualized.

Results

The influence of four different hybridization times (t = 15, 30, 60, 120 min) and temperatures (T = 40, 60, 70, 75 °C) on the fluorescence signals of major and minor binding sites of two α-satellite DNA-probes (D12Z2, D8Z2) was examined (Ta-

Table I. This table shows the average numbers of minor binding sites (± standard deviation) for different hybridization times and temperatures of the two repetitive α-satellite DNA probes D8Z2 and D12Z2. The values were calculated on the basis of 30 metaphase spreads each, selected by random access.

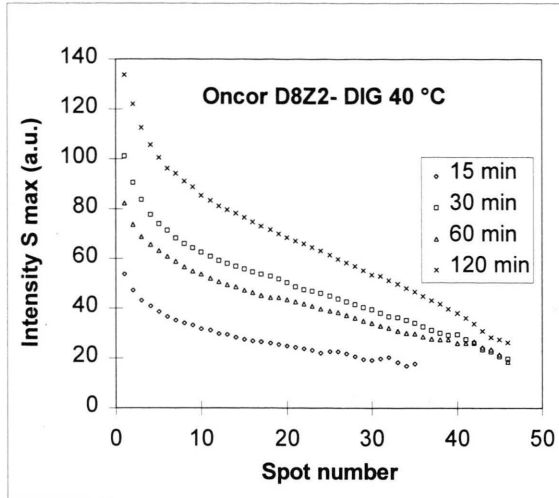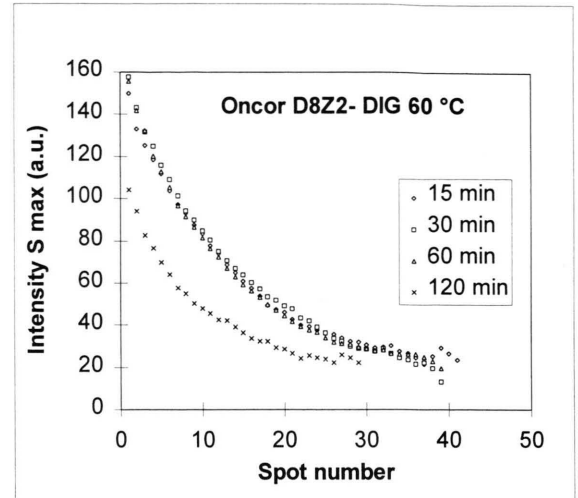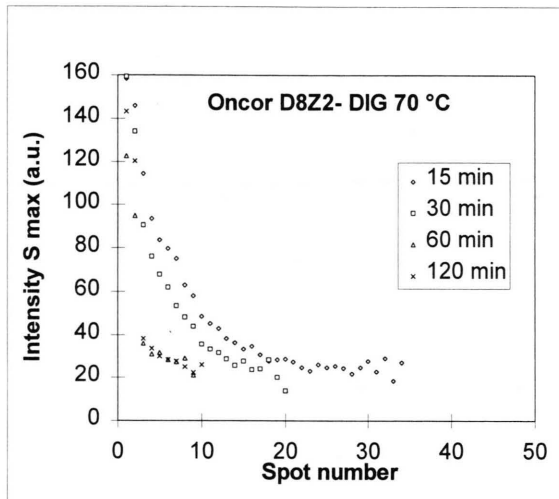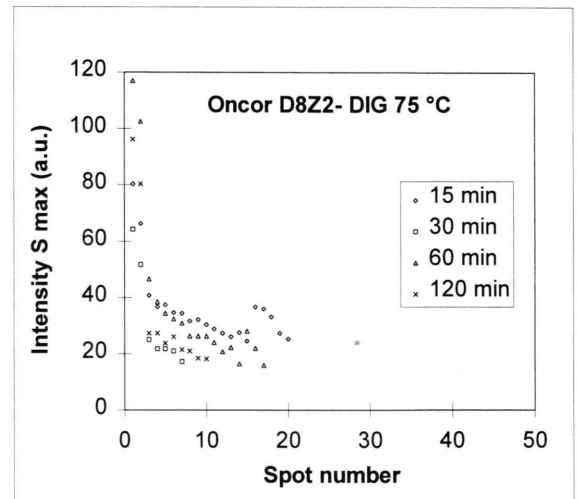| | Incubation time | | | |
	15 min	30 min	60 min	120 min
D8Z2				
40 °C	23.9 ± 7.2	40.4 ± 3.2	38.6 ± 5.7	42.1 ± 3.0
60 °C	28.4 ± 6.3	29.9 ± 4.4	29.5 ± 5.0	17.5 ± 6.0
70 °C	18.8 ± 7.2	9.0 ± 4.5	2.1 ± 2.1	2.9 ± 2.5
75 °C	4.8 ± 5.1	1.2 ± 1.6	5.4 ± 3.9	1.5 ± 2.0
D12Z2				
40 °C	17.6 ± 6.9	20.8 ± 4.9	26.6 ± 9.0	38.5 ± 4.3
60 °C	13.3 ± 1.2	19.6 ± 3.0	16.3 ± 3.4	15.1 ± 1.9
70 °C	12.7 ± 1.4	11.6 ± 2.6	9.8 ± 3.8	14.7 ± 1.2
75 °C	–	0.3 ± 1.0	0.3 ± 0.5	0.1 ± 0.3

Fig. 1a.

Fig. 1b.

Fig. 1c.

Fig. 1d.

Fig. 1. S_{max} vs. spot no. with decreasing values (D8Z2). Each point represents the average of 30 metaphase spreads. Each graph a) $T = 40$ °C, b) $T = 60$ °C, c) $T = 70$ °C, and d) $T = 75$ °C contains four curves ($t = 15$ min, $t = 30$ min, $t = 60$ min, $t = 120$ min).

ble I, Figs. 1, 2). For $T = 40$ °C the number of fluorescent spots on the chromosomes increased with t so that for $t = 120$ min on average about 42 chromosomes per metaphase spread (= major + minor binding sites) were labeled in their centromeres. Those hybridization conditions were found to be useful for all centromere labeling with these probes.

For $T = 60$ °C the average number of minor binding sites was approximately constant (within statistical errors) for all t but in most cases it was

lower than for $T = 40$ °C. For $T = 70$ °C the number of minor binding sites was further reduced with a minimum at $t = 60$ min. Although the lowest numbers of minor binding sites were found at $T = 75$ °C, this turned out not to be the optimum condition because the signal intensity decreased considerably and the morphological appearance of the chromosomes became poor for this high hybridization temperature. Especially the D12Z2 probe became problematic under these conditions. In Table I the average numbers of minor binding sites
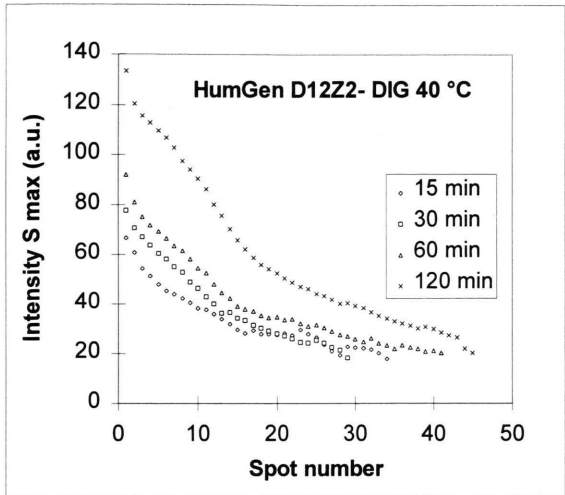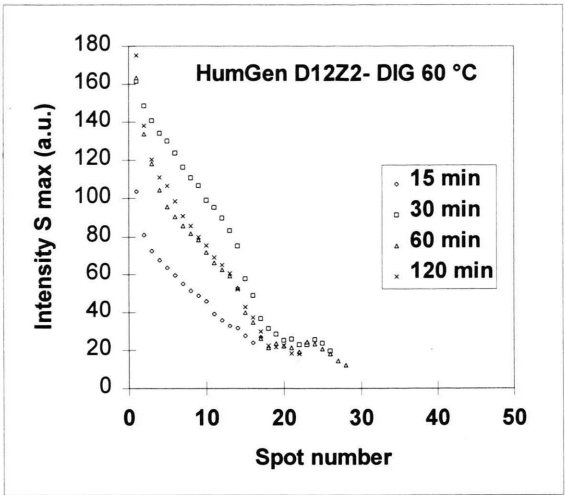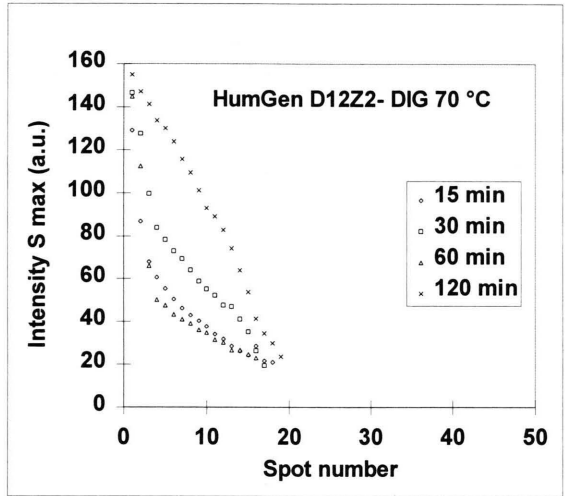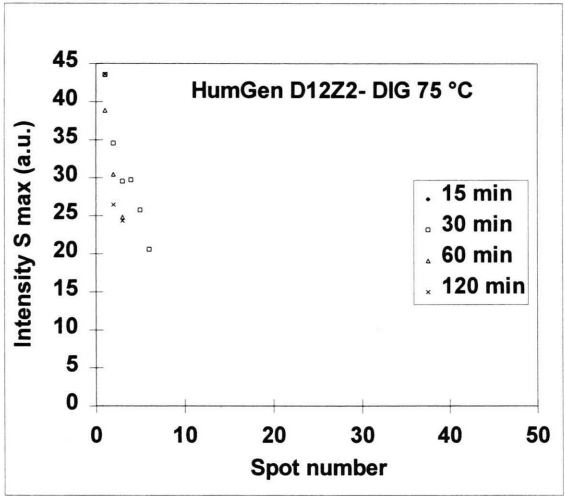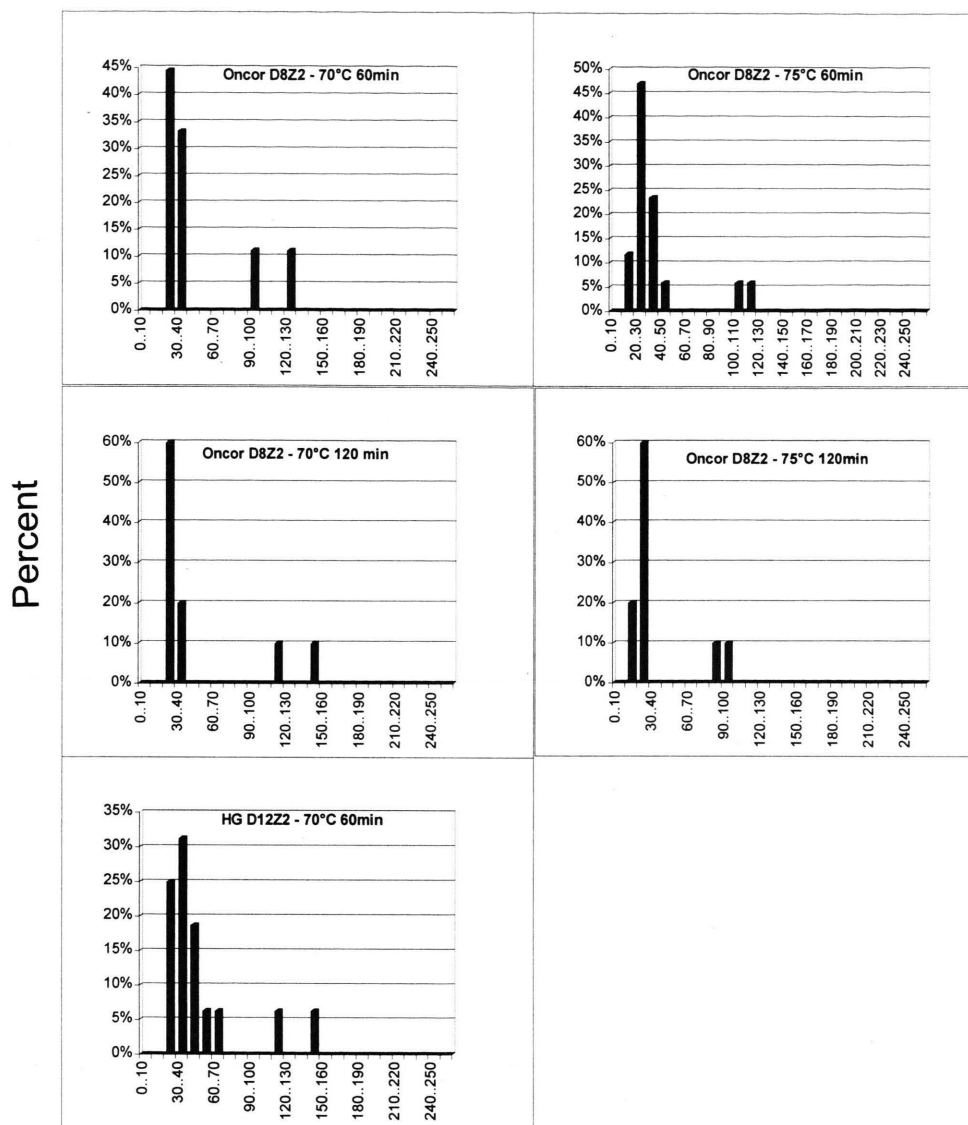
Fig. 2a.

Fig. 2b.

Fig. 2c.

Fig. 2d.

Fig. 2. S_{max} vs. spot no. with decreasing values (D12Z2). Each point represents the average of 30 metaphase spreads. Each graph a) $T = 40\,°C$, b) $T = 60\,°C$, c) $T = 70\,°C$, and d) $T = 75\,°C$ contains four curves ($t = 15$ min, $t = 30$ min, $t = 60$ min, $t = 120$ min).

under the given hybridization conditions are summarized.

For all different hybridization conditions, S_{max} (maximum intensity), A (spot area) and S_{int} (integrated intensity $S \times A$) of all registered FISH spots of a metaphase spread were calculated and arranged according to decreasing values. This means that spot number 1 and 2 correspond to the presumptive major binding sites. For each case the S_{max}-values for spot 1, 2, 3 ... etc. were averaged using 30 metaphase spreads of one (T, t) condition.

In all cases S_{max} and S_{int} showed well comparable results, i.e. a high S_{max} corresponded to a high S_{int} and a low S_{max} to a low S_{int}. Fig. 1 shows the results for the D8Z2 probe and Fig. 2 for the D12Z2 probe. In Fig. 3, histograms of the mean values (see Figs. 1 and 2) for optimal hybridization conditions are given. For $T = 70\,°C$ the intensities of spot 1 and 2 were considerably higher than for all other spots (except 120 min for D12Z2 – see Fig. 2c). The same result was obtained for $T = 75\,°C$. For the D8Z2 probe the optimal hybridiza-

Intensity Distribution

Fig. 3. Relative frequencies of integrated mean spot intensities for $T = 70$ °C and $t = 60, 120$ min (D8Z2, D12Z2); and $T = 75$ °C and $t = 60, 120$ min (D8Z2 only).

tion conditions (= best discrimination of major and minor binding sites by S_{max}) were obtained for $T = 70$ °C and $t = 60$ min and $t = 120$ min, respectively.

For D12Z2, similar results were obtained for $T = 70$ °C and $t = 60$ min. In contrast to D12Z2, the D8Z2 probe also showed a reasonable discrimi-

a)

$$S_{max,n}$$

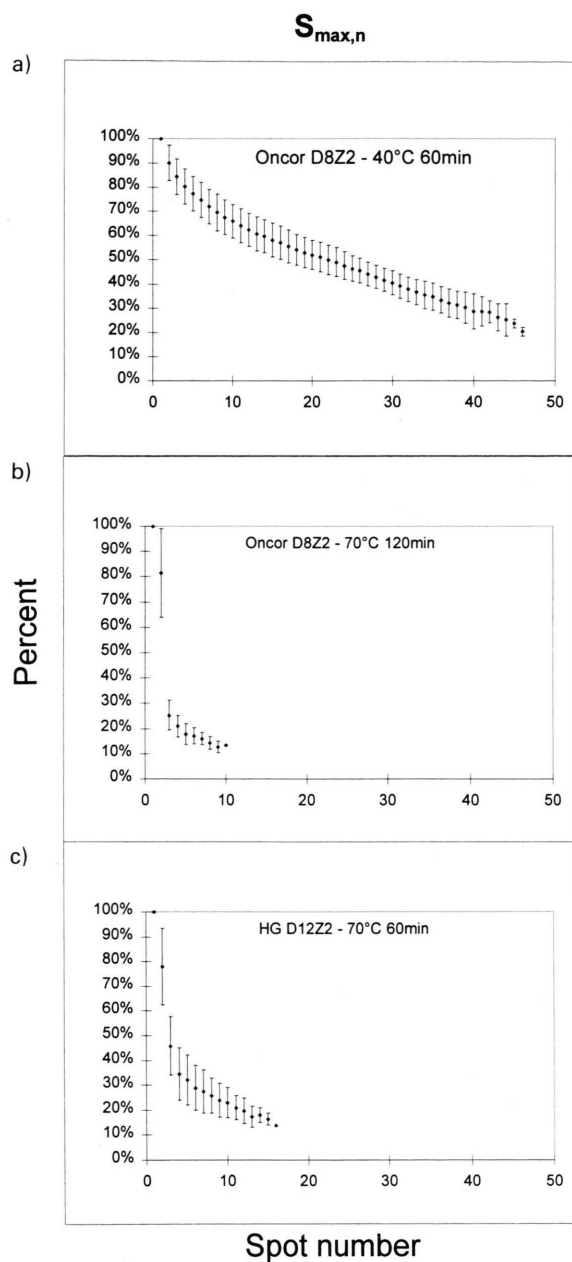b)

Percent

c)

Spot number

Fig. 4. S_{max}^{norm} (normalized S_{max} values) vs. spot no. with decreasing values. Each point represents the mean value from 30 metaphase spreads. The bars indicate the standard deviation σ a) D8Z2 for $T = 40\,°C$ and $t = 60$ min; b) D8Z2 for $T = 70\,°C$ and $t = 120$ min; c) D12Z2 for $T = 70\,°C$ and $t = 60$ min.

nation of major and minor binding sites for $T = 75\,°C$ with $t = 60$ and 120 min.

The absolute intensity values varied from experiment to experiment. In addition, intercellular intensity variations in metaphase spreads on the same microscope slide were found. To account for that, normalized intensity values S_{max}^{norm} were calculated (normalization to the brightest spot in each metaphase spread) for the corresponding spots of 30 randomly chosen metaphase spreads. Accordingly, the brightest spot in each metaphase spread obtained the value $S_{max}^{norm} = 100\%$. All other spots obtained smaller values according to their normalized spot intensities. For each of these smaller values, the mean of S_{max}^{norm} and its standard deviation σ was determined. From the mean S_{max}^{norm} and its standard deviation σ an estimate of the significance of discrimination of major and minor binding sites for the "optimized" hybridization conditions was obtained (Fig. 4). The low renaturation temperature resulted in a staining of all centromeres. In this case, in the individual cells, no significant discrimination of the two major binding sites from other binding sites was possible. In contrast to this result, high temperature hybridization conditions discriminated the two brightest spots (major binding sites) from all other spots (minor binding sites) within a range of more than one standard deviation σ.

Discussion

"Non formamide protocols" (omitting formamide and other denaturing organic chemical agents) have been introduced to accelerate the FISH procedure. This greatly improves the possibility to study systematically the effect of different hybridization conditions. Here we investigated the influence of hybridization temperature (T) and time (t) on the stringency. The problem of minor binding sites due to low stringency conditions was overcome by appropriate hybridization conditions of Fast-FISH. Hybridization time and temperature revealed to be sensitive parameters to influence the result for the two highly repetitive α-satellite probes used.

Low hybridization temperatures and increased hybridization times resulted in fluorescence labels on nearly all centromeres (low stringency). This "low stringency" condition does not allow the

identification of specific chromosomes. However, an optimization of such conditions in order to completely label all centromeres may be useful for the application of the Fast-FISH procedure in biological dosimetry. Labeling of centromeres of a specific chromosome type allows the rapid detection of dicentric chromosomes (Cremer *et al.*, 1990). Labeling of all centromeres in principle is expected to allow the detection of all dicentric chromosomes in a metaphase spread (Durm *et al.*, manuscript in preparation).

In this report, the opposite aim was persued. The hybridization conditions were modified in such a way, that with one image parameter S_{max} of the labeling spots a clear identification of the major binding sites was possible. The hybridization temperatures used were around 70 °C. In spite of that, the signal intensities were sufficient to allow useful hybridization times between 1 and 2 hours. Additionally, the high stringency option for Fast-FISH reported here has been extended to a variety of other repetitive probes (Haar *et al.*, 1996; Durm *et al.*, manuscript submitted). The

combination of Fast-FISH and automated digital image analysis offers an attractive method for a quantitative chromosome analysis in all cases where routine measurements in a short time have to be performed. For general screening purposes a standard set of probes may be used. Optimized values for T and t can be found so that the computer analysis can be completely automatized. The results suggest that a special non-formamide protocol may be used for specific labeling and to suppress considerably chromosomal binding of highly repetitive probes to minor binding sites. However, it is anticipated that especially in tumor diagnostics, single copy probes have to be included depending on the problem to be studied. Thus to study the influence of time and temperature quantitatively in detail may offer further aspects of Fast-FISH to the application of other than repetitive probes (Durm *et al.*, manuscript submitted).

Acknowledgement

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