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Optimized Fast-FISH with α-satellite probes: acceleration by microwave activation

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Abstract

It has been shown for several DNA probes that the recently introduced Fast-FISH (fluorescence *in situ* hybridization) technique is well suited for quantitative microscopy. For highly repetitive DNA probes the hybridization (renaturation) time and the number of subsequent washing steps were reduced considerably by omitting denaturing chemical agents (e.g., formamide). The appropriate hybridization temperature and time allow a clear discrimination between major and minor binding sites by quantitative fluorescence microscopy. The well-defined physical conditions for hybridization permit automatization of the procedure, e.g., by a programmable thermal cycler. Here, we present optimized conditions for a commercially available X-specific α-satellite probe. Highly fluorescent major binding sites were obtained for 74ºC hybridization temperature and 60 min hybridization time. They were clearly discriminated from some low fluorescent minor binding sites on metaphase chromosomes as well as in interphase cell nuclei. On average, a total of 3.43 ± 1.59 binding sites were measured in metaphase spreads, and 2.69 ± 1.00 in interphase nuclei. Microwave activation for denaturation and hybridization was tested to accelerate the procedure. The slides with the target material and the hybridization buffer were placed in a standard microwave oven. After denaturation for 20 sec at 900 W, hybridization was performed for 4 min at 90 W. The suitability of a microwave oven for Fast-FISH was confirmed by the application to a chromosome 1-specific α-satellite probe. In this case, denaturation was performed at 630 W for 60 sec and hybridization at 90 W for 5 min. In all cases, the results were analyzed quantitatively and compared to the results obtained by Fast-FISH. The major binding sites were clearly discriminated by their brightness.

Key words
- Fluorescence *in situ* hybridization
- Fast-FISH
- Metaphase chromosomes
- α-Satellite DNA probes
- Microwave treatment
- Quantitative image analysis

Introduction

Fluorescence *in situ* hybridization (FISH) has become a widespread technique in cytogenetics for specific chromosome labelling. In addition to DNA probes of higher complexity, repetitive DNA probes such as α-satellite probes are routinely used in chromosome research, clinical diagnosis, or radiation biology (1-6). A broad spectrum of these probes in combination with hybridization reaction kits is commercially available. These reaction kits, as well as most of the established FISH protocols (7), are based on developments from earlier *in situ* hybridization procedures (8-10). In these protocols,

denaturing chemical agents are usually applied to both the DNA probe and the chromosomal target. Especially the use of formamide at high concentrations combined with moderate heat treatment has been established ("formamide protocol"). This treatment is also used to control stringency conditions, i.e., minor binding sites of repetitive probes can be suppressed by appropriate addition of formamide so that ideally only one (= the specific) binding site remains labelled. However, the consequences of using formamide are 1) a long hybridization time (typically overnight up to several days (11) or even many days (12)) for the amounts of probe DNA usually applied, followed by 2) a complex, workload washing procedure.

Hybridization time and hybridization temperature are used here to indicate the time and temperature required for probe-target DNA renaturation only.

In routine clinical diagnosis or biological dosimetry (3,5,6), it is highly desirable not only to accelerate the entire hybridization procedure but also to develop protocols that are simple and reproducible. It has been observed (13) that FISH of repetitive DNA probes is also feasible in the absence of formamide or equivalent denaturing chemical agents. Starting from these findings, a modified FISH technique ("non-formamide protocol") with a high temperature denaturation treatment has been described (14,15). For highly repetitive DNA probes (pUC 1.77, D8Z2, D12Z1, D15Z1 and p82H which are specific for regions on the human chromosomes 1, 8, 12, 15 and all chromosomes, respectively) it was possible to reduce the hybridization time considerably (15-30 min). This technique is called Fast-FISH.

The incorporation of fluorescein-labelled nucleotides into the DNA probes has reduced the number of subsequent washing steps to one so that the complete FISH procedure, i.e., the preparation of microscopy ready slides, can be completed in less than one hour. Due to the low stringency conditions, minor binding sites also became visible. For pUC 1.77 and D15Z1, major and minor binding sites were discriminated by computer image analysis only. For this purpose, a classification algorithm based on spot intensity and spot area evaluation was written. Although this approach led to reasonable results in many cases, it was desirable to further optimize the hybridization parameters and the FISH procedure regarding reproducibility. In the "non-formamide protocol", formamide and other denaturing agents have been eliminated. Therefore, hybridization time and hybridization temperature acquire a more important role as two highly sensitive basic parameters for the hybridization process. Additional factors of still unknown significance might be, for example, the state of condensation and aging of the chromosomal targets, the consistence and the pH of the buffer, and the type of chemical modifications used to label the DNA probes. For D8Z2 and D12Z1 it was shown by systematic experiments how the two major parameters, hybridization time and hybridization temperature, influence the hybridization behavior of α-satellite probes, and how this effect can be used to easily discriminate major and minor binding sites. The optimal hybridization conditions concerning rapidity and quality of chromosome morphology were obtained using a hybridization temperature of 70°C and a hybridization time of 60 min. Under these conditions, major and minor binding sites were clearly discriminated by the maximum intensity of the corresponding FISH spots (16). On the other hand, low hybridization temperatures (about 40-50°C) permitted the complete labelling of all centromeres (6,17). These conditions may be useful for the application of Fast-FISH in biological dosimetry in order to highlight dicentric chromosomes.

Since the thermal conditions (temperature, time) are sensitive parameters that influence the hybridization result, a computer-controlled thermal cycler was introduced in

order to adjust the optimal thermal conditions during the procedure (18).

Microwave activation for FISH has been recently suggested by Soloview et al. (19). Microwaves are commonly used in immunochemistry to fix and stabilize tissue prior to embedding and cutting or for antigen retrieval and immunoincubations (20). For a successful application, however, specialized laboratory ovens were used. In the present study, we show that a common commercially available microwave oven is a practical alternative to a thermal cycler for Fast-FISH. Comparable results were obtained for chromosome 1- and X-specific α-satellite DNA probes. In addition, the complete Fast-FISH procedure was accelerated.

Material and Methods

Preparation of slides and DNA probes

Metaphase chromosomes and cell nuclei were obtained from human lymphocytes isolated from peripheral blood and fixed on microscope slides with methanol/acetic acid by standard techniques (21,22). For chromosome labelling, a digoxigenin-11-dUTP-labelled X-chromosome-specific α-satellite probe and a digoxigenin-11-dUTP-labelled chromosome 1-specific α-satellite probe (pUC 1.77) commercially available from Boehringer Mannheim (FRG) were used.

In situ hybridization

Specimen preparation

Approximately 20 ng of the labelled DNA probe, 1 µl hybridization buffer (10x: 100 mmol/l Tris-HCl, 30 mmol/l $MgCl_2$, 500 mmol/l KCl, 10 mg/l gelatine, pH 8.3, 20ºC) and 1 µl 20 x SSC were diluted in deionized H_2O to a final volume of 10 µl. The hybridization mixture was pipetted onto the microscope slides with metaphase spreads. The slides were then covered with a coverslip and sealed with rubber cement (Fixogum, Marabu, Tamm).

Denaturation and hybridization with a thermal cycler

The slides were placed on a specially designed working plate of a thermal cycler (Cyclogene HL-1, Thermo-DUX GmbH, Weiterstadt, FRG) as usually done for the polymerase chain reaction. Thermal denaturation was performed at 95ºC for 5 min. Hybridization was performed at 72ºC (chromosome 1 probe) or 74ºC (X chromosome probe) for 60 min. The thermal cycler reduced the operating temperature from 95ºC to 72ºC (74ºC) in less than 1 min.

Microwave-activated denaturation and hybridization

The slides were mounted on a small glass filled with 100-120 ml water and placed in the center of the circling plate of a commercially available microwave oven (Sharp R-3E44). Denaturation was performed at 630 W for 60 sec or 40 sec (chromosome 1 probe), or at 900 W for 20 sec (X chromosome probe) followed directly by hybridization at 90 W for 5 min (chromosome 1 probe) or 4 min (X chromosome probe).

Detection

After hybridization the coverslips were carefully removed and the slides were incubated in a washing buffer (1 x PBS, pH 7.2, + 0.2% Tween 20) for 5 min at room temperature. For fluorescence labelling with antidigoxigenin-fluorescein Fab fragments (Boehringer Mannheim), the stock solution of 200 µg/ml was diluted in 1 x PBS, pH 8.4 (Boehringer Mannheim) to a final concentration of 10 µg/ml. Approximately 70 µl of this solution was pipetted onto each slide. The slides were covered again with a plastic coverslip and placed in a humidified steel

chamber which was incubated at 37°C for 1 h. After removing the coverslips, the slides were washed in 1 x PBS, pH 7.2, for 2 min in the dark and the chromosomes were counterstained with propidium iodide (0.2 µg/ml). After air drying at 40°C, the slides were mounted with Vectashield Mounting Medium to reduce photo bleaching of the dyes during image acquisition.

Digital image analysis

For visualization, an image analysis system described in detail elsewhere (23) was used. The system is based on a fluorescence microscope (Leitz Orthoplan, Leica, Wetzlar, FRG) equipped with a Plan APO 63X/NA 1.40 objective and a tube magnification of 1.25X. Excitation with a 50-W mercury arc lamp was performed 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, about 25 metaphase spreads and about 25 nuclei were recorded using a cooled color CCD-camera (CF 15 MC, Kappa, Gleichen, FRG). A constant acquisition time for each slide was chosen. The recorded 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) was run on a PC (80486) under WINDOWS 3.1 with the Novel DOS operating system. In this software package, a program subroutine was implemented which was designed for automated spot finding and evaluation. All quantitative data of the FISH spots were obtained from the green image plane of the RGB-image. The program automatically analyzed all regions of high intensity. All spots with an intensity value below an experimentally given threshold (calculated by the signal/noise ratio) were excluded from evaluation. 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 maximum intensity, the borderline of the spot was fixed. For all FISH spots, S_{max} (maximum intensity), S (averaged intensity), F (area) and S/R (signal to noise) 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, the values obtained from the different metaphase spreads were averaged and the mean values and standard deviations were computed for the other spots. These data were further processed by a standard spread sheet program and visualized.

Results

The optimal hybridization conditions (time and temperature) were systematically examined for the α-satellite DNA probe specific for the X chromosome. Using a thermal cycler, an optimum of 74°C hybridization temperature and of 60 min hybridization time was determined. Under these conditions, the number of minor binding sites was very low and they were clearly discriminated from the two major binding sites (female cells) by their fluorescence intensity or by the area of the labelling sites (Figure 1A). For 30 metaphase spreads, the average number of binding sites was 3.43 ± 1.59, and for 75 cell nuclei it was 2.69 ± 1.00. Figure 2 shows the results for S_{max}, and the normalized intensity to the brightest spot. These results are compatible with earlier investigations on α-satellite DNA probes of chromosomes 1, 8, and 12 (16,18).

For the same X DNA probe, the experimental conditions were systematically optimized using a household microwave oven. It was important to place the specimen slide onto a glass filled with water in order to prevent “overcooking” and to maintain the chromosome morphology. Since the micro-

wave field in this oven is not homogeneous, the best position of the slide had to be determined. In this case it was in the center of the circling plate and raised a few centimeters. At this position the best denaturation conditions were 900 W for 20 sec followed by 4 min hybridization at 90 W (Figure 1B). The average number of binding sites was 4.36 ± 1.91 for 25 metaphase spreads and 1.81 ± 0.66 for 25 cell nuclei. These results agreed with the values obtained from the thermal cycler experiments within the error ranges. The fluorescence intensities S_{max} were lower than in the thermal cycler experiments so that the intensity gap between major and minor binding sites was less pronounced (Figure 3a, left and right panels). Normalization to the brightest intensity, however, resulted in a discrimination between major and minor binding sites by more than one standard deviation (Figure 3b, left and right panels). A considerable intensity gap between major and minor binding sites was found (Figure 3c, left and right panels).

Comparing Figures 2 and 3, it is obvious that the number of binding sites in the metaphase spreads was always higher than in the cell nuclei. The intensity values of the major binding sites always showed a larger variability in the metaphase spreads than in the cell nuclei. Considering the absolute intensity, a clear discrimination between major and minor binding sites could only be

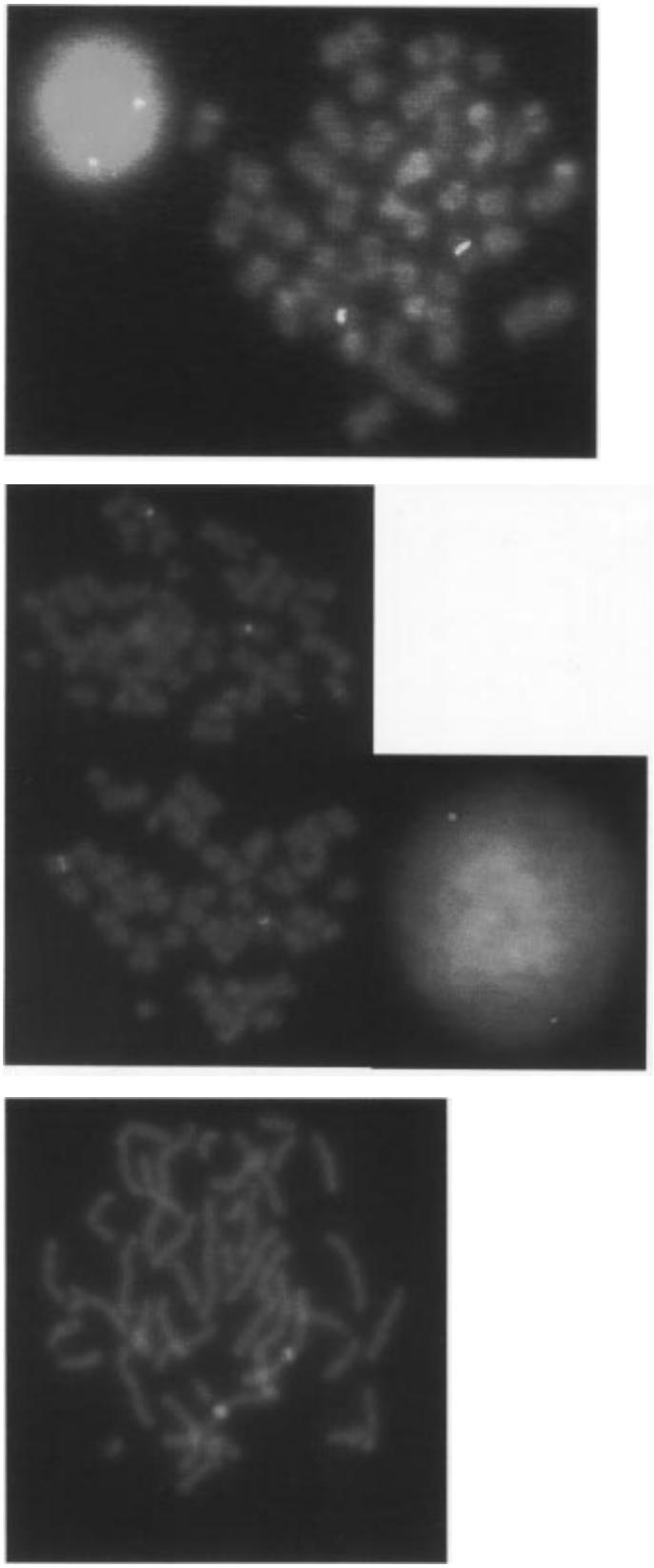

Figure 1 - Digitized images of female metaphase spreads and interphase cell nuclei after labelling with α-satellite DNA probes specific for the X chromosome and for chromosome 1. All spots with an intensity value below an experimentally given threshold (calculated by the signal/noise ratio) were excluded from evaluation. *A*, X chromosome labelling by thermal cycler treatment (denaturation at 95ºC for 5 min and hybridization at 74ºC for 60 min); *B*, X chromosome labelling by microwave treatment (denaturation at 900 W for 20 sec and hybridization at 90 W for 4 min); *C*, chromosome 1 labelling by microwave treatment (denaturation at 630 W for 40 sec and hybridization at 90 W for 5 min). Magnification 63X, tube magnification 1.25X.

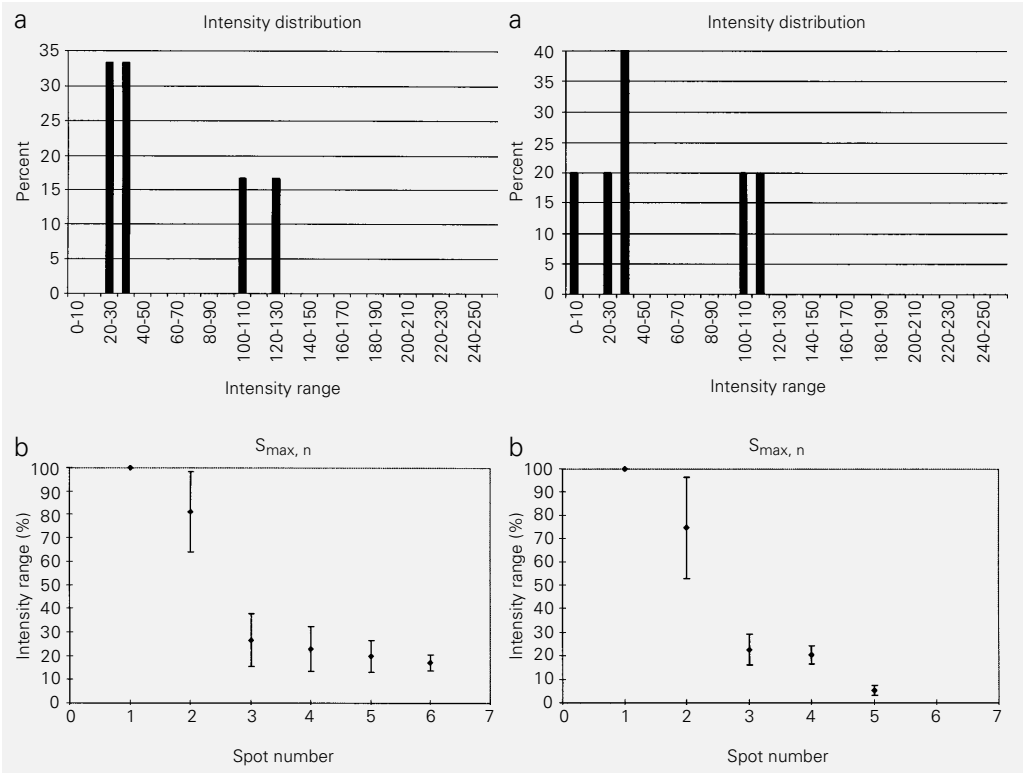

Figure 2 - Intensity distributions of the labelling sites of the α-satellite DNA probe specific for the X chromosome (female human lymphocytes) after thermal cycler treatment (hybridization time 60 min). *Left panels*, Results averaged from 30 metaphase spreads (average number of binding sites 3.43 ± 1.59). *Right panels*, Results averaged from 75 cell nuclei (average number of binding sites 2.69 ± 1.00). *a*, Relative frequency of labelling sites in percent versus intensity S_{max} (arbitrary units). The two bars of high intensity represent the two major binding sites. The minor binding sites (bars of lower intensity) are clearly discriminated by an intensity gap. *b*, Normalized intensities (normalized to the brightest spot = 100%) versus spot numbered according to decreasing intensity. The error bars show the standard deviation. The two major binding sites are discriminated from the minor binding sites by about four standard deviations of the brightest minor binding site.

made for the thermal cycler hybridization. In the case of the microwave oven hybridization the discrimination was enhanced after normalization to the brightest intensity spot.

The results obtained for the X-chromosome-specific probe were confirmed by experiments using a chromosome 1 α-satellite DNA probe (Figure 1C). For 60-sec denaturation at 630 W and 5-min hybridization at 90 W in the same position in the microwave oven, 3.11 ± 0.69 binding sites were found on average on 25 metaphase spreads and 2.20 ± 0.68 binding sites in 25 cell nuclei. A significant discrimination in intensity (more than one standard deviation) was again found between major and minor binding sites of metaphase spreads (data not shown). For the cell nuclei, comparable results were not obtained under these conditions. However, a reduction of the denaturing time from 60 sec to 40 sec solved this problem. Here, 3.31 ± 1.49 binding sites were found on 25 metaphase spreads and 2.30 ± 0.97 in 25 cell nuclei. These results agree with experiments using the thermal cycler, where 3.48 ± 1.42 binding sites were found on metaphase spreads and 2.60 ± 0.95 in cell nuclei (18). As demonstrated for the X chromosome, major and minor binding sites were separated by a gap in the absolute intensity S_{max} as well as in the normalized intensity (Figure 4).

Discussion

"Non-formamide protocols" (omitting denaturing chemical agents) have been introduced to accelerate the FISH procedure and to reduce the workload of post-treatment specimen washing (14,15). Hybridization time and temperature are very sensitive parameters in this protocol influencing the "stringency", i.e., the number and fluorescence intensity of minor binding sites. For α-satellite DNA probes, a low number of less fluorescent minor binding sites were found for a hybridization temperature within the range of 70°C and for a short hybridization

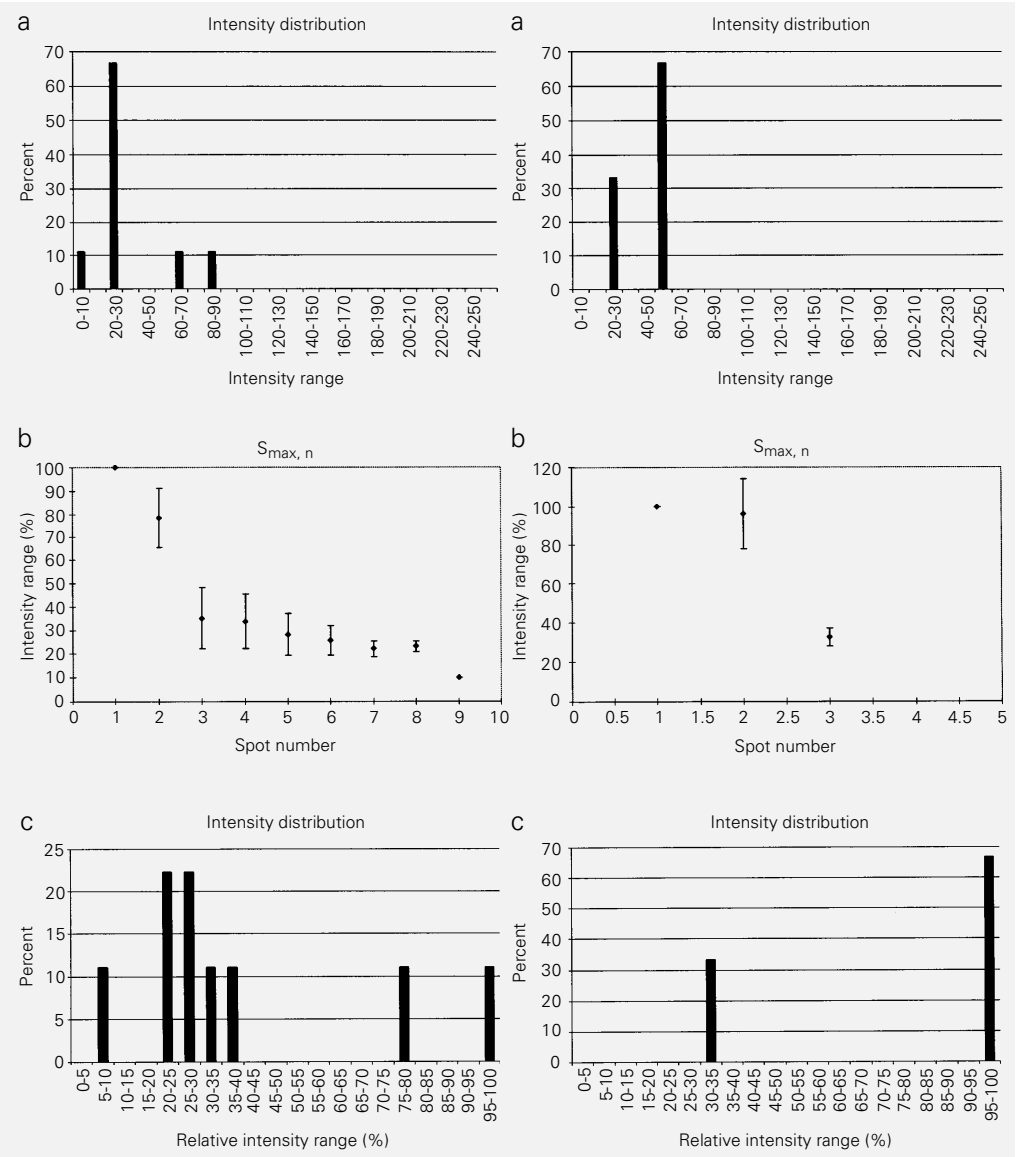

Figure 3 - Intensity distributions of the labelling sites of the α-satellite DNA probe specific for the X chromosome (female human lymphocytes) after microwave-activated hybridization (hybridization time 4 min). *Left panels*, Results averaged from 25 metaphase spreads (average number of binding sites 4.36 ± 1.91). *Right panels*, Results averaged from 25 cell nuclei (average number of binding sites 1.81 ± 0.66). *a*, Relative frequency of labelling sites in percent versus intensity S_{max} (arbitrary units). For the metaphase spreads, two bars for the two major binding sites are visible (intensity range 60-70, 80-90). In the cell nuclei, both major binding sites are in the intensity range of 50-60 (right bar). *b*, Normalized intensities (normalized to the brightest spot = 100%) versus spot numbered according to decreasing intensity. The error bars show the standard deviation. Major and minor binding sites are discriminated by about four standard deviations. *c*, Shows the results from the same experiment in a frequency bar plot. The large intensity gap between major and minor binding sites is clearly visible. Note that in Figure 3c (right panel) the intensity values of the two major binding sites fit the same intensity range, in contrast to Figure 3c (left panel), where the intensities of the two homologue chromosomes show larger variation.

time (60 min) (16,18). A reduction of the hybridization temperature to 40-50°C as well as a longer hybridization time resulted in a larger number of minor binding sites and in an increase of their fluorescence intensity. Consequently, this protocol was useful to simultaneously highlight all centromeres (17).

For a better adjustment of the temperatures and the temperature gradient of denaturation and hybridization, a modified thermal cycler appeared to be useful (18). The results shown here confirm the reliability of this technique. Although thermal cyclers are nowadays standard equipment for DNA probe amplification by the polymerase chain reaction (24), specially designed working plates for microscope slides are not always available.

Here, we have presented a practical, "low cost" alternative based on the use of a common, household microwave oven. In addition, Fast-FISH was further accelerated by reducing the time of denaturation and hybridization. Using a microwave oven, two points have to be considered carefully: the

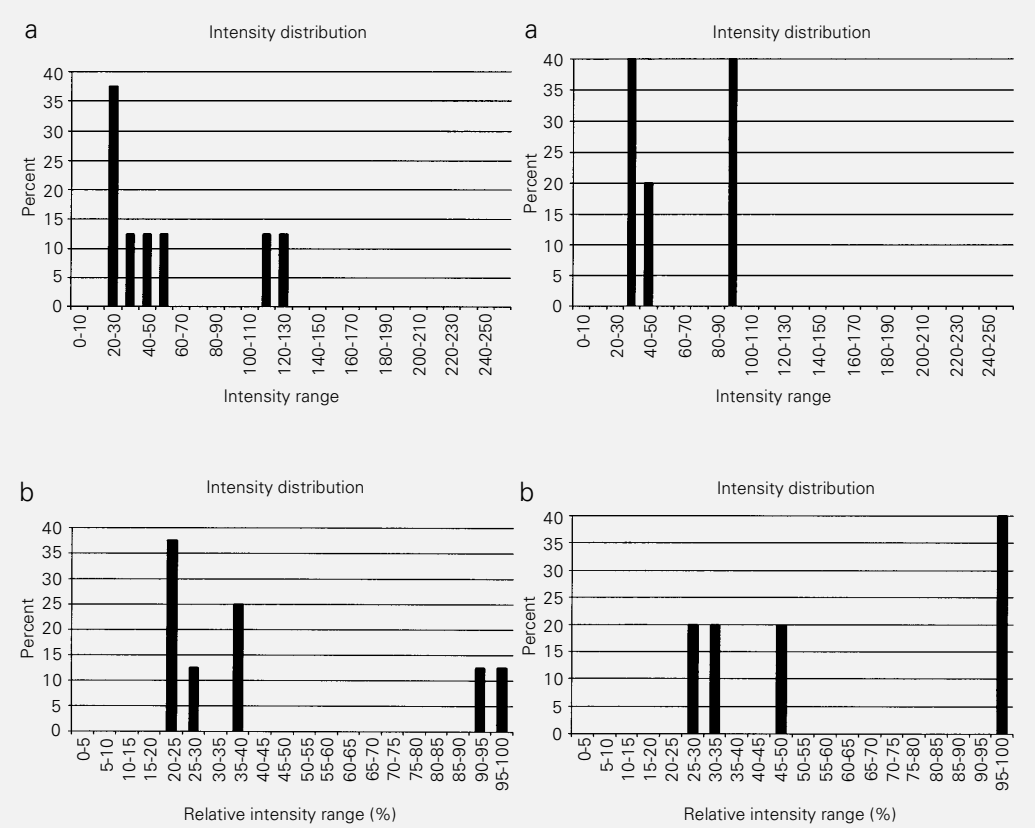

Figure 4 - Intensity distributions of the labelling sites of the α-satellite DNA probe (pUC 1.77) specific for chromosome 1 after microwave-activated hybridization (hybridization time 5 min). *Left panels*, Results averaged from 25 metaphase spreads (average number of binding sites 3.31 ± 1.49). *Right panels*, Results averaged from 25 cell nuclei (average number of binding sites 2.30 ± 0.97). *a*, Relative frequency of labelling sites in percent versus intensity S_{max}; *b*, relative frequency of labelling sites in percent versus normalized intensity.

amount of water to avoid specimen "cooking" and the optimal position in the microwave field inside the oven. In our experience, the conditons were optimized after only a few test experiments.

With respect to low-power hybridization (90 W), it should also be taken into consideration that a microwave oven usually reduces its average irradiation power by appropriate changes in pulses of the full power microwave peak exposure. Thus, the pulse frequency has to be measured to find the appropriate hybridization time. For a given microwave oven, these pre-experiments have to be performed only once so that for routine FISH this workload is negligible.

The mechanism and the reason for the accelerated labelling are not well understood. The hybridization buffer contains H_2O which can be optimally heated in a microwave oven. Thus, local thermal effects may contribute to DNA denaturation. In addition, the diffusion velocity of the DNA probe may be modified. Obviously, microwave treatment exerts a considerable influence on proteins. This suggests that chromosomal proteins may also be involved in the *in situ* hybridization mechanism. To answer this question will be the task of future investigations.

In most cases, highly repetitive DNA probes have been investigated thus far and the Fast-FISH protocols have been optimized for them. The possibility to paint chromosomes has been recently shown (25). However, to what extent the Fast-FISH protocol described here may be extended to single copy probes or complex probes deserves further quantitative studies.

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