Non-Enzymatic, Low Temperature Fluorescence *in situ* Hybridization of Human Chromosomes with a Repetitive α-Satellite Probe

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In all DNA-DNA *in situ* hybridization (ISH) procedures described so far in the literature, the production of single-stranded target DNA sequences plays a decisive role. This can be achieved either by enzymatic treatment at physiological temperatures or by the separation of double-stranded DNA sequences. Denaturation by heat and chemical agents (e.g. formamide) is regarded as a prerequisite for the non-enzymatic ISH process. However, additional mechanisms of a non-enzymatic ISH procedure are conceivable which do not require high temperature treatment combined with formamide. Here, we report on a non-enzymatic, non-formamide, low temperature, fluorescence *in situ* hybridization (FISH) procedure which allowed a microscopic visualization and quantitative fluorescence analysis of the binding sites of a repetitive DNA probe. Following only probe denaturation at 94 °C, hybridization was performed at 52 °C for 30 min, i.e., at nearly physiological temperatures. Moreover, increasing the hybridization time to 3 hours indicated that hybridization sites became also visible at 37 °C. Since the protocols are based on recently described Fast FISH developments, the technique will be called Low Temperature Fast-FISH (LTFF).

Introduction

In situ hybridization (ISH) has become a powerful technique to localize nucleic acid sequences in cell nuclei or in metaphase chromosomes. Fluorescence labelling of the hybridized sites (i.e. Fluorescence *in situ* Hybridization (FISH)) has found widespread applications in cytogenetics (for review see: Lichter *et al.*, 1991; Trask, 1991; Cremer and Cremer, 1992; Lichter and Cremer, 1992; Gray *et al.*, 1994; Cremer *et al.*, 1995a). Its sensitivity has improved considerably, allowing the detection of only a few kilobases up to the delineation of whole chromosomes using complex DNA probes established from flow sorted chromosomes.

For ISH, (chemically modified) probe sequences are bound in a sequence specific way to target sequences present *in situ* in metaphase chromosomes and chromatin of cell nuclei. Usually the chromosomes and cell nuclei are fixed on glass slides.

The principle mechanism of DNA-DNA ISH can be described as the specific annealing of a DNA probe to complementary DNA sequences of the target material followed by the visualization of the location of the probe (e.g. FISH). In non-enzymatic procedures, it is generally acknowledged that both, probe *and* target DNA sequences have to be separated into single-stranded sequences. After this denaturation process, the single-stranded DNA probe specifically hybridizes under stringent conditions to the complementary single-stranded sequences on the target DNA forming a new double-stranded molecule that incorporates the label.

In standard hybridization protocols the thermally induced denaturation process is accompanied by the use of chemical denaturing agents such as formamide. These agents decrease the melting temperature of double-stranded DNA isolated in solution. Under the assumption that similar effects of denaturing agents should occur in chromosomal DNA or in nuclear chromatin, respectively, such

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compounds are used to increase the stringency of DNA-probe binding. Recently, new FISH techniques (Fast-FISH) have been developed that omit formamide or other equivalent denaturing chemical agents in the hybridization buffer (Celeda *et al.*, 1992; 1994; Haar *et al.*, 1994). This Fast-FISH technique has the particular advantage that the hybridization time (renaturation time) can be reduced to about 15–30 min using similar amounts of probe DNA as in conventional (i.e. formamide) FISH (Durm *et al.*, 1996; 1997). The temperatures for denaturation and specific renaturation, however, are considerably different compared to the usually applied formamide ISH protocols. Because of the lack of denaturing chemical agents, the denaturation process of probe and target DNA is only performed thermally. Systematic studies of hyperchromicity effects (Rauch, Wolf, Durm, Hausmann, Cremer, manuscript in preparation) of DNA solutions and chromosome suspensions showed that in this case strand separation effects are detectable in temperature ranges above 80 °C. In the case of highly repetitive DNA probes, renaturation time and temperature influenced the number of minor binding sites. It was shown that for a couple of α-satellite probes hybridization temperatures of about 60 °C or 70 °C had the advantage that the major binding sites were clearly identified by their intensity. Only very few less intense minor binding sites were visible. With quantitative image analysis it was easy to significantly classify the binding sites according to one intensity parameter (Durm *et al.*, 1996; Haar *et al.*, 1996).

However, the drawbacks of all present methodology consist in a loss of native chromosomal structure by either chemical (standard FISH protocol) or thermal (Fast-FISH protocol) denaturation. To overcome this problem it was investigated whether thermal denaturation of only the DNA probes would be sufficient to assure specific probe binding to chromosomes at ambient temperatures. As first experiments with a low temperature, non-enzymatic in situ hybridization technique showed (Kraus *et al.*, 1995), it is possible to perform in situ hybridization experiments under physiological temperature conditions. With respect to magnetic separation (Hartig *et al.*, 1995) of labelled chromosomes, this hybridization technique was done in suspension with isolated metaphase chromosomes. Only the probe DNA was

thermally denatured and annealed to the metaphase chromosomes.

In this report, a non-enzymatic, low temperature *in situ* hybridization protocol without the use of chemically denaturing agents was studied for fixed human chromosomes on slides (low temperature Fast-FISH = LTFF). A systematic quantitative analysis was made for different hybridization parameters. The LTFF-protocol was applied to a highly repetitive DNA probe (pUC1.77) for fluorescence labelling of the 1q12 region on human lymphocyte metaphase spreads and interphase nuclei, respectively. Only the probe was thermally denatured at 94 °C and put afterwards onto the prewarmed target. The influence of different hybridization times and temperatures was investigated.

Material and Methods

Preparation of human metaphase spreads on slides

Metaphase chromosomes were obtained from human lymphocytes isolated from peripheral blood by standard techniques (Arakaki and Sparks, 1963). The lymphocytes were stimulated by Phytohemagglutinin M (2.5 µg/ml lymphocyte medium) and cultivated for 72 hours followed by a Colcemid block (27 µM) (Boehringer Mannheim, Mannheim, FRG) for the last two hours. The cells were treated according to a modified hexandiol method and the metaphase spreads were fixed on slides by means of methanol/acetic acid (3:1, v:v) (Moorhead *et al.*, 1960). The slides were stored in 100% ethanol until use. Prior to use, they were rinsed with 100% ethanol and air dried.

Preparation of the human DNA probe pUC1.77

The chromosome 1 subcentromeric highly repetitive DNA probe pUC1.77 (commercially available from Boehringer Mannheim) was labelled *in vitro* in the presence of digoxigenin-11-dUTP by means of nick translation of the entire plasmid pUC1.77 (Cooke *et al.*, 1979) by the manufacturer. According to the product information, the DNA lengths typically varied around 200–500 bp.

Labelling of human metaphase spreads by LTFF

20 ng of the labelled DNA probe, 1 µl hybridization buffer (10×: Tris-HCl 100 mmol/l; MgCl₂

30 mmol/l; KCl 500 mmol/l; gelatine 10 mg/l; pH 8.3 (20 °C)) and 1 μl 20× SSC were diluted in deionized H_2O to make up a final volume of 10 μl in a 0.5 ml Eppendorf tube. Thermal denaturation of only the probe DNA was performed in a waterbath at 94 °C for 5 min.

For hybridization, the slides with the metaphase spreads and cell nuclei were placed onto a plate of a thermocycler specially designed for *in situ* hybridization experiments (Cyclogene HL-1, thermo-DUX GmbH) (Haar *et al.,* 1996, Durm *et al.,* 1997). The slides were preheated to the following temperatures of 72 °C, 62 °C, 52 °C, 42 °C and 37 °C for 5 min. The hybridization mixture with the DNA probe was pipetted on the slides which were covered by a cover glass and sealed with rubber cement (Fixogum, Marabu, Tamm). Hybridization took place at 72 °C, 62 °C, 52 °C, 42 °C for 30 min and 37 °C for 3 hours.

Visualization of the hybridization sites on human metaphase spreads

The coverslips were carefully removed and the slides were incubated in the washing buffer (1×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 (200 μg/ml) was diluted in 1× PBS (pH 8.4) to a final concentration of 10 μg/ml. Approximately 70 μl of this solution were pipetted on each slide, which was covered with a plastic coverslip and placed into a humidified steel chamber. The closed chamber was incubated in a waterbath at 37 °C for 1 hour. Then the slides were washed again in 1× PBS (pH 7.2) for 2 min in the dark. The chromosomes were counterstained with propidium iodide (0.2 μg/ml). After air drying in a prewarmed chamber at 37 °C the slides were mounted with Vectashield mounting medium (Serva, Heidelberg, FRG).

Microscopy and evaluation

For visualization, an image analysis system was applied. It is based on a fluorescence light microscope (Leitz Orthoplan, Leica, Wetzlar, FRG) equipped with a Plan APO 63× /NA 1.40 objective and a 50 W mercury arc lamp. The fluorescein labelled and propidium iodide counterstained metaphase spreads and interphase nuclei were excited via a band pass filter (450−490 nm) and detected via a 515 nm long pass filter. On the slides, metaphase spreads and interphase nuclei were chosen by random access. For each hybridization temperature and each hybridization time, 25 metaphase spreads and 25 interphase nuclei were recorded using a cooled colour CCD-camera (CF 15 MC, Kappa Meßtechnik, Gleichen, FRG) with 1.25× post magnification (Bornfleth *et al.,* 1996). A constant acquisition time for each slide was chosen. The images were transferred to a colour frame grabber (ITI Vision Plus Colour CFG 512; Kappa Meßtechnik). For registration and automated evaluation, the commercially available software package OPTIMAS (BioScan, Edmonds, WA, USA) was used running on a PC (80486) under MS WINDOWS 3.1 with the operating system Novell DOS 7. 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-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 metaphase spreads and interphase nuclei, respectively, S_{max} (maximum intensity) and A (area) were computed. In addition, the normalized intensity values S_{max_n} (normalized to the intensity of the brightest FISH spot in each metaphase spread / interphase nucleus) were calculated. For each experiment, the 25 randomly chosen metaphases and cell nuclei, respectively, were averaged and the mean values and standard deviations were computed for the other spots. All these data were further processed by a standard spread sheet program and visualized.

Results

The influence of six different hybridization conditions (72 °C-30 min, 62 °C-30 min, 52 °C, 30 min, 42 °C-30 min, 37 °C-30 min, 37 °C-3hours) on the fluorescence signals of the pUC1.77 probe was examined. In Table I the average numbers of binding sites under the given hybridization conditions are summarized.

Table I. Average numbers of binding sites ($\pm$ standard deviation) for six different hybridization conditions. The values were calculated on the basis of 25 metaphase spreads and interphase nuclei each, selected by random access.

Metaphases	37 °C	42 °C	52 °C	62 °C	72 °C
30 min	n.d.	n.d.	2.8 ± 0.6	2.3 ± 0.5	2.4 ± 0.8
180 min	2.2 ± 1.1	–	–	–	–
Nuclei	37 °C	42 °C	52 °C	62 °C	72 °C
30 min	n.d	n.d.	2.1 ± 0.6	2.0 ± 0.0	2.1 ± 0.3

n.d. = not detectable.

For all different hybridization conditions, S_{max} (maximum intensity), A (spot area) and S_{int} (integrated intensity S×A) of all registered FISH spots of metaphase spreads and interphase nuclei were calculated and arranged according to decreasing values. This means that spot 1 and 2 correspond to the presumptive major binding sites. For each case, 25 metaphase spreads or 25 interphase nuclei of one hybridization condition, the S_{max} values for spot 1, 2, 3 .. etc. were averaged. In all cases, S_{max} and S_{int} showed well comparable results.

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_n} and their standard deviations were calculated (normalization to the brightest spot in each metaphase spread or interphase nucleus) (Fig. 1).

For 72 °C target temperature and 30 min hybridization time, the fluorescence signals on the metaphase spreads were very bright and clearly visible. The amount of minor binding sites was very low (mean number of all binding sites 2.4 ± 0.8). The few minor binding sites which occurred were clearly distinguishable from the major binding sites (Fig. 1a). These results agreed well to results obtained by Fast-FISH with denaturation of both, probe and target DNA (Durm *et al.*, 1996). Similar results were obtained for the interphase nuclei; the signal intensity and the number of minor binding sites was a little bit lower (Fig. 1e).

The experiments with target temperatures of 62 °C and hybridization time of 30 min showed also clearly detectable fluorescence signals although the absolute signal intensity appeared to

be a little bit lower compared to 72 °C (Fig. 1b). On the other hand the chromosomal morphology seemed to be preserved better. The interphase nuclei showed no minor binding sites (Fig. 1f).

For 52 °C target temperature and 30 min hybridization time (Fig. 1c), the number of binding sites on the metaphase spreads increased slightly (2.8 ± 0.6). In the case of the interphase nuclei, a clear discrimination between major and minor binding sites, using the intensity parameter only, became more difficult (Fig. 1g).

No significant fluorescence signals were detectable for the hybridization temperatures 42 °C and 37 °C at 30 min hybridization time. In another experiment, in which the hybridization was performed under physiological temperature conditions (37 °C) and with a longer hybridization time of 3 hours, a clearly detectable result was obtained (Fig. 1d). As the example shows, two fluorescence signals were clearly visible on the two homologous chromosomes 1 (Fig. 2). Although the absolute signal intensity was not very high, it was sufficient for digital image recording with the CCD-camera system.

Discussion

A new, low temperature, non-enzymatic in situ hybridization technique (LTFF) that is based on the Fast-FISH technique (Celeda *et al.*, 1994; Durm *et al.*, 1996) was introduced. The aim of this report was the systematic and quantitative analysis of LTFF under different parameters (hybridization temperature and hybridization time) in order to find optimal conditions to discriminate major from minor binding sites. In contrast to Fast-FISH protocols so far published, only the DNA probe was thermally denatured. The results were well comparable to those obtained by Fast-FISH with high temperature denaturation of both target and probe DNA.

The Fast-FISH procedures described so far have the disadvantage that due to the high temperature during denaturation the native structure of the chromosomal proteins may not be preserved. A conservation of the native structures of chromosomal proteins as well as other proteins, however, appears to be desirable in various cases. For example, it seems to be of great use in the preservation of nuclear fine structure for investigations of
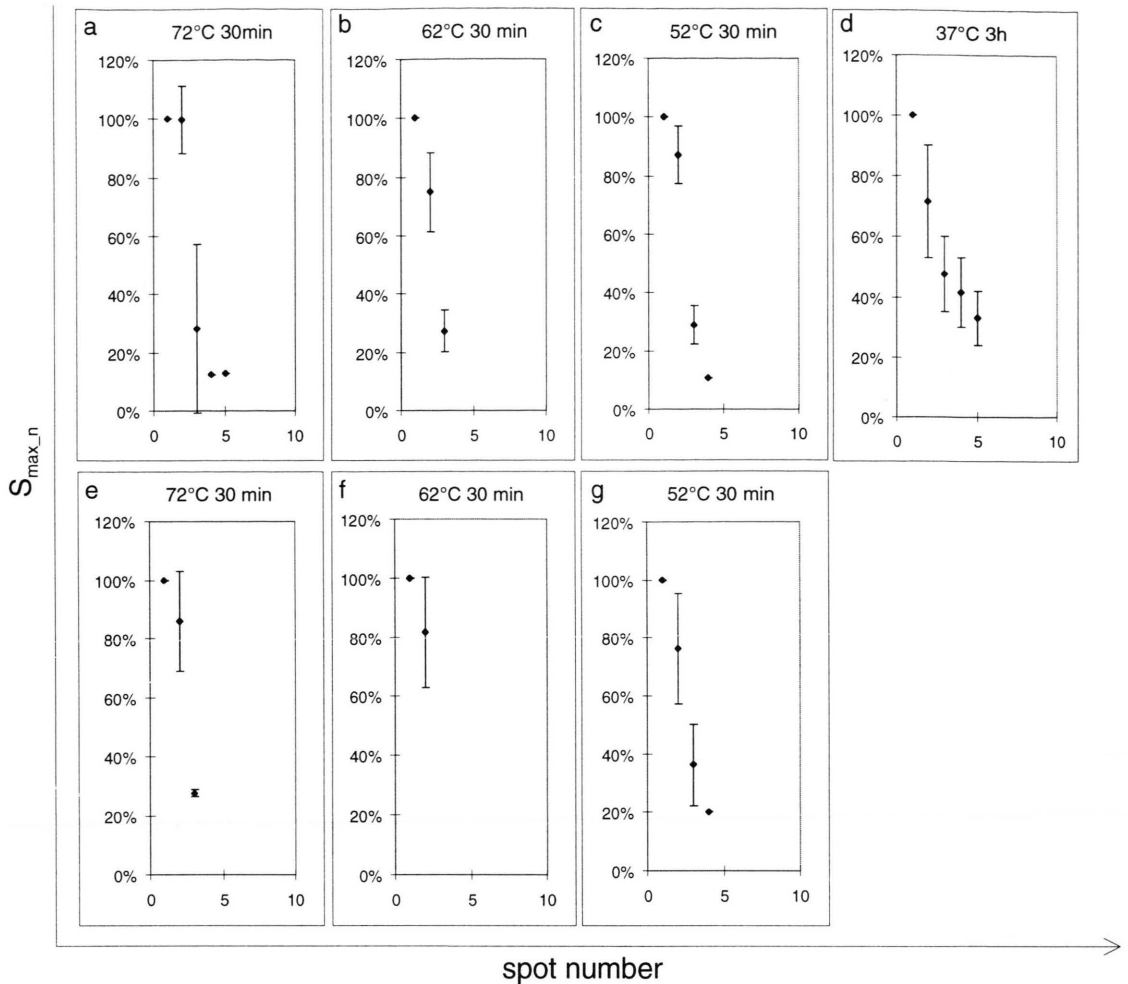
Fig. 1. S_{max_n} (normalized S_{max} values) vs. spot number with decreasing values. For each graph, 25 metaphase spreads or cell nuclei, respectively, were evaluated:
metaphase spreads (a) 72 °C/30 min, b) 62 °C/30 min, c) 52 °C/30 min, d) 37 °C/3hours and interphase nuclei, (e) 72 °C/30 min, f) 62 °C/30 min, g) 52 °C/30 min).
The bars indicate the standard deviation (The highest S_{max_n} value [100%] has no standard deviation because of normalization. Lower S_{max_n} values without a bar denote that spots with these values were observed very rarely.)

nuclear architecture (Cremer *et al.*, 1993; 1995b); other useful applications might be a better preservation of isolated chromosomes following FISH-labelling for the use in slit-scan flow cytometry (Hausmann *et al.*, 1991), or a facilitated combination of antibody binding and FISH for medical applications.

As the results for the highly repetitive DNA probe pUC1.77 show, an optimal hybridization temperature was between 52° and 72 °C. With this probe reasonable results were obtained after 30 min of hybridization. It was shown that nearly

stringent conditions (almost no minor binding sites) were possible for higher hybridization temperatures (62 °C). Although the target was not thermally denatured separately, these hybridization temperatures may include a thermal denaturation. In addition, the preparation of the slides, especially low pH pre-treatment as methanol/acetic acid fixation, may contribute to chromosome denaturation. However, systematic studies by hyperchromicity measurements (Rauch, Wolf, Durm, Hausmann, Cremer, manuscript in preparation) showed that major conformation changes of chro-
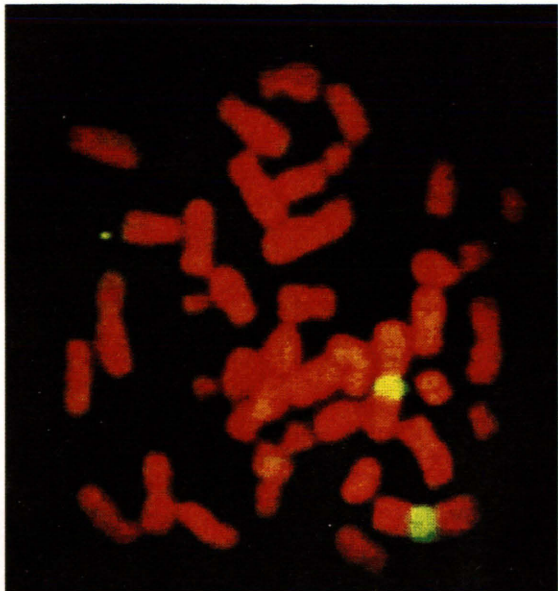
Fig. 2. Example of a metaphase spread after 3 hours hybridization time and a target temperature of 37 °C, using the LTFF-procedure.

mosomes corresponding to denaturation effects were less influenced by pH reduction, in contrast to pure DNA in solution. DNA solutions and chromosome suspensions showed a completely different thermal denaturation behaviour under the influence of denaturing chemical agents (e.g. formamide). The strong reduction of the maximum melting temperature typical for free DNA in solution was not found for entire chromosomes in solution. These results suggested that below 72^0C the conditions for heat denaturation of double-stranded chromosomal DNA may not be fulfilled at all. Thus, the results presented here suggest that, compared to the commonly accepted mechanism (i.e. single-stranded probe DNA hybridizes with single-stranded target DNA to form double-stranded labelling couples), alternative mechanisms for ISH may exist.

For example, small amounts of single-stranded target DNA present either naturally, or as the result of the fixation process alone, might in some cases be sufficient to allow visualization of probe-target binding sites using low temperature protocols. A second alternative might be the indirect binding of a DNA probe sequence to a protein bound to double-stranded target DNA in a se-

quence specific manner. Although this would not be an *in situ* hybridization in its strict sense, it would be equivalent to ISH in its practical use. As a third possibility, the formation of triple DNA strands from single-stranded and double-stranded DNA strands (especially in the low temperature ranges of ISH) or complex, mixed DNA conformations may also be considered (Felsenfeld *et al.*, 1957; Lee *et al.*, 1987; Burkholder *et al.*, 1988; Moffat, 1991).

The mechanism of single-stranded probe DNA coupling with the double-stranded target DNA to a triple DNA strand appears to be possible both from theoretical considerations and experimental evidence. This would be compatible with the experiments under „physiological" temperature conditions where a specific binding of the pUC1.77 probe was obtained. If a triple helical configuration is assumed, such highly repetitive sequences would be preferred as binding sites. Therefore, the possibility of ISH with a single-stranded DNA probe to double-stranded target DNA in chromosomes or cell nuclei cannot be rejected purely on theoretical grounds. Accordingly, a „low temperature" hybridization process should be possible where the target is exposed only to maximum temperatures well below the melting point for double-stranded DNA. The reason why so far a non-enzymatic, low temperature ISH protocol has not been described might be attributed to the common use of denaturing chemical agents such as e.g. formamide. These agents form H-bonds with DNA bases and thus may be expected to lower the stability of triple-stranded DNA to such a point that their formation under the usually used hybridization conditions is not longer possible.

It is anticipated that LTFF conditions below 40 °C reveal a considerable reduction in the fluorescence intensity of the binding sites. To overcome these problems, quantitative image analysis on the basis of high sensitive CCD-camera systems may be useful. Further progress in image evaluation may be obtained by time gated fluorescence life time spectroscopy (Schneckenburger *et al.*, 1994).

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