Painting of Human Chromosome 8 in Fifteen Minutes

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Rapid Chromosome Painting, FAST-FISH

The technique of chromosome-*in-situ* suppression (CISS)-hybridization (chromosome painting) has now been well established. However, all standard protocols so far require long renaturation times (typically 12 hours and more). Here, we describe a new, extremely fast protocol for chromosome painting using a commercially available, directly fluorescence labelled probe for chromosome 8. The hybridization conditions used omit separate preannealing procedures and denaturing chemical agents. The renaturation time required for chromosome painting was reduced to 15 minutes. In addition, most washing steps were eliminated. As a consequence, the entire painting procedure was feasible in less than half an hour.

Introduction

The routinely applied protocols for fluorescence *in situ* hybridization (FISH) of metaphase chromosomes and cell nuclei (for review see: Lichter *et al.,* 1991, Trask 1991, Cremer and Cremer 1992, Lichter and Cremer 1992) with specific DNA probes make intensive use of denaturing chemical agents for the treatment of both the probe and the target DNA. Especially formamide in high concentrations (50%–70%) is used in combination with moderate heat denaturation (referred to as "Formamide Protocol"). 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 chemical agents. Starting from these preliminary findings, a modified FISH-technique called Fast-FISH has been described (Celeda *et al.,* 1994, Haar *et al.,* 1994). The applicability of this "Non-Formamide Protocol" to chromosome painting by CISS hybridization (Lichter *et al.,* 1988, Cremer *et al.,* 1988, Pinkel *et al.,* 1988) or to comparative genomic hybridization (Kallioniemi *et al.,* 1992, du Manoir *et al.,* 1993) appeared to be difficult.

For several highly repetitive DNA probes, however, it was shown that without using the "Formamide Protocol", it was possible to shorten the hybridization time considerably, i.e the time for DNA probe-target renaturation (down to typically 15–30 minutes). Hybridization time and hybridization temperature became two prominent parameters to control the stringency of probe binding (Durm *et al.,* 1996; Haar *et al.,* 1996). Fluorescence microscopy in combination with appropriate hybridization parameters allowed to quantify major and minor binding sites. Additional parameters of still unknown significance might be the consistence and pH of the buffer and the type of chemical modification used to label the DNA-probes.

Here, we show that the "Non-Formamide Protocol" in combination with appropriate hybridization parameters allows a sufficiently expressed sequence specific labelling of chromosomes and suppression of the chromosomal binding of highly repetitive sequences to make possible rapid chromosome painting.

Materials and Methods

Metaphase spreads 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 chromosome medium) and cultivated for 72 h followed by a Colcemid block (27 µM) for 2 h. The cells were further treated according to a protocol described elsewhere (Moorhead *et al.,* 1960) with slight modifications. After hypotonic treatment the cells were fixed on slides by methanol/acetic acid (3:1, v:v).

For fluorescence *in situ* hybridization, the commercially available chromosome painting kit for chromosome 8 from GIBCO (Life Technologies,

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Eggenstein, FRG) was used. According to the protocol of the manufacturer, this probe specifically paints chromosome 8 under standard ("Formamide Protocol") conditions. The DNA probe was directly fluorescence labelled with SpectrumOrange (red fluorescence). The probe was delivered in a buffer without denaturing agents as for instance formamide.

In situ hybridization was performed as follows: 1 µl of the labelled DNA probe mixture ready to use, 2 µl hybridization buffer [10 x : 100 mmol/l Tris-HCl, 30 mmol/l $MgCl_2$, 500 mmol/l KCl, 100 µg/ml gelatine, pH 8.3 (20^0 C)], and 2 µl 20 x SSC were diluted in deionized H_2O. The hybridization mixture (20 µl) was pipetted on the microscope slides, which were covered by a cover glass and sealed with rubber cement. The slides were placed in a specially designed, closed stainless steel chamber for 6 min denaturation at 94^0C. Hybridization took place at 62^0C for 15 min. For DNA counterstaining, DAPI (5 µmol/l) was used.

For visualization, a fluorescence microscope (Leitz Orthoplan) was applied with an image acquisition setup described elsewhere (Celeda *et al.*, 1994; Bornfleth *et al.*, 1996). A PlanAPO 63 x / 1.40 NA objective and appropriate filters were used to obtain two separate images for each metaphase spread ("red" = SpectrumOrange labelling signal; "blue" = DAPI counterstaining signal). For image acquisition, a cooled color CCD camera (CF 15 MC, Kappa, Gleichen, FRG), a color frame grabber, and a 80486 PC were used. The image processing was performed with standard routines of the commercially available software package OPTIMAS (BioScan, Edmonds, WA, USA). Contrast enhancement was performed in the blue image plane. After thresholding, the background was eliminated in the red image plane. The thresholds were chosen individually for each metaphase spread (see also Fig. 2).

Results and Discussion

Fig. 1 shows two typical metaphase spreads after chromosome painting of chromosome 8 using the "Non-Formamide Protocol" with a hybridization time of 15 min. After the hybridization procedure the morphology of the chromosomes was preserved as judged by microscopic observation. In the painted metaphase spreads two chromosomes of equal size and centromere location were observed carrying considerable high red signals (Table I). According to the relative chromosome length as compared to the longest chromosome (presumptive chromosome 1) and according to the centromere index, the relative length and centromere location of these chromosomes were compatible with chromosome 8.

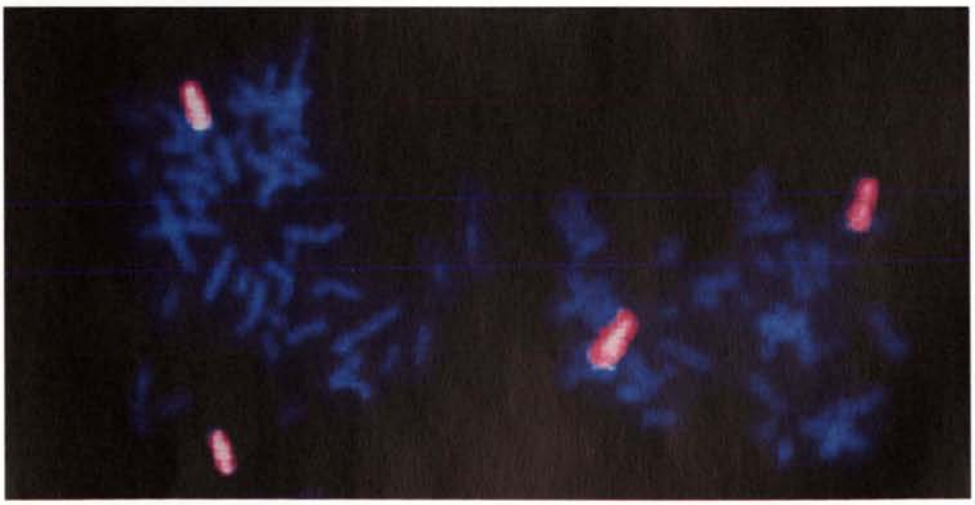

Fig. 1 Digital color images of two metaphase spreads hybridized with a chromosome 8 paint probe labelled with SpectrumOrange and counterstained with DAPI. The renaturation time was 15 min. The blue (DAPI) and red (SpectrumOrange) images were overlayed and thresholded as described in the text.

Table I. Quantitative results of evaluated metaphase spreads obtained from the red image plane only. a) The mean intensity value, the standard deviation, and the area of the two labelled chromosomes calculated after thresholding are given in comparison to the mean intensity "background" on the other chromosomes of a metaphase spread. b) Graphical representation of the mean intensity data of a). The considerable difference of labelled and non labelled material is well visible from the height of the columns.

a)

No.	1. Chromosome Intensity	Std. dev.	Area	2. Chromosome Intensity	Std. dev.	Area	Background
1	214	34	332	202	29	299	112
2	208	22	280	187	17	324	79
3	197	33	299	190	24	287	65
4	197	28	305	176	27	326	94
5	183	35	296	181	31	318	88
6	174	34	242	164	21	231	82

b)

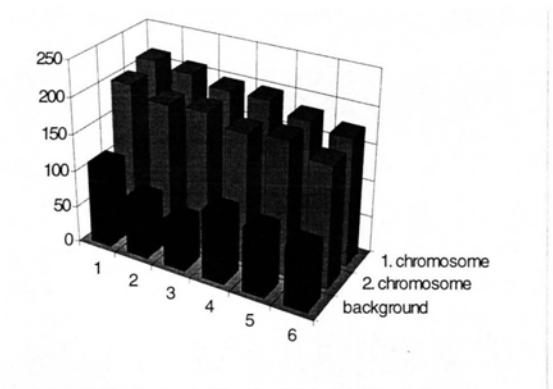

Fig. 2 shows gray value distributions obtained from the red image plane of six metaphase spreads also evaluated for Table 1. These intensity distributions were acquired along manually chosen straight lines through the given metaphase spread crossing the two labelled chromosomes (compare Celeda *et al.*, 1994). The locations of the hybridized chromosomes were clearly visible as two maximum intensity (gray value) peaks. The distinct difference between the hybridization signals and the unspecific background on the other chromosomes was used to fix the applied threshold resulting in images as for instance shown in Fig. 1. In all cases evaluated, the shape of the intensity profiles confirmed the visual impression by which real hybridization labelling was distinguished from unspecific "entanglement".

The results indicate that choosing the appropriate conditions for the probe concentration, the buffer, the hybridization time and the hybridization temperature, the "Non-Formamide Protocol" allows rapid chromosome painting. Compared with the conventional (Formamide) painting protocols (hybridization times typically 12 hours and more), an extreme reduction in the entire preparation time for the hybridization process was achieved. A separate preannealing step was not necessary. Chromosome specific painting became possible in less than half an hour. Systematic studies accompanied by detailed quantitative image analysis are required to further study the influence of the individual parameters of the process. Finally, it is anticipated that not only the painting of individual chromosomes using chromosome

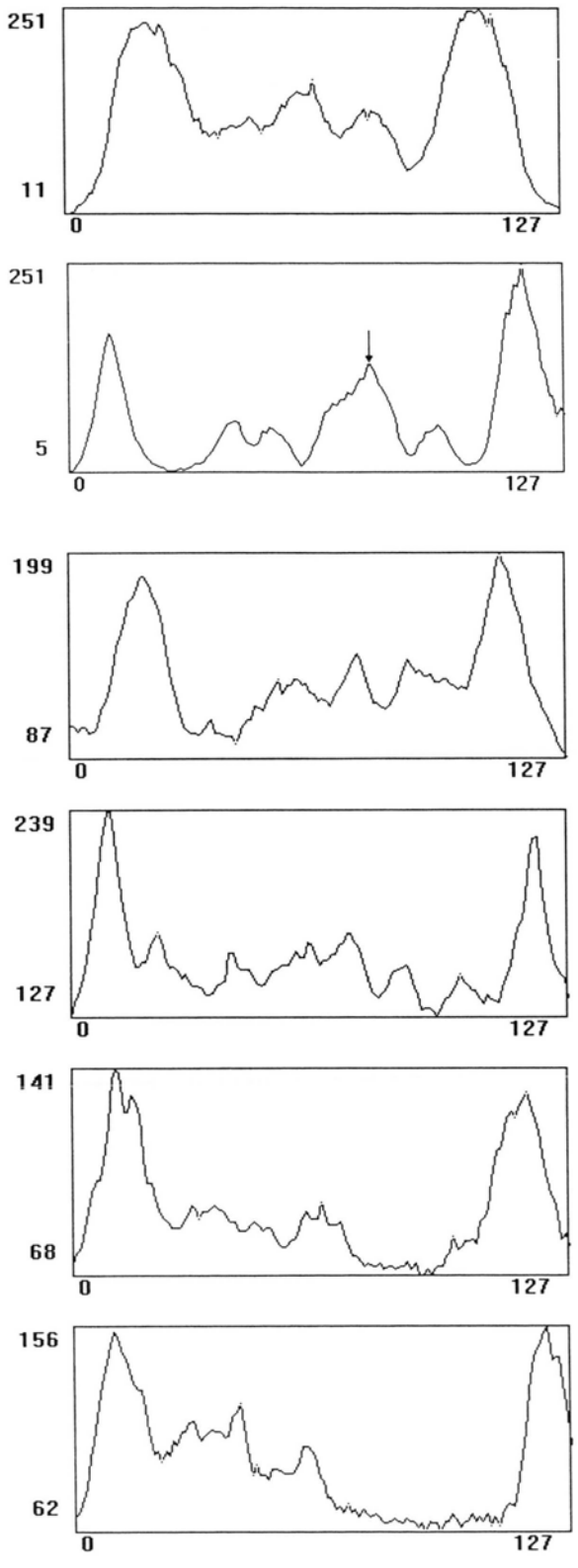

Fig. 2. Gray value profiles along individually chosen straight lines in the red image planes of six analyzed metaphase spreads. The peaks indicate the chromosomes crossed through the line. In all cases the two chromosomes labelled by in situ hybridization according to the "Non-Formamide Protocol" were represented by the two highest peaks (the relatively high peak indicated by arrow was due to overlapping chromosomes identified to be unlabelled by visual observation). The considerable difference of the two peaks to the remaining "background" peaks on the other chromosomes was used to fix the threshold for image visualization and evaluation (for comparison see Fig.1).

specific DNA libraries may be accelerated by about one order of magnitude. From the physicochemical mechanisms involved in rapid fluorescence (Non-Formamide) *in situ* hybridization, it may be speculated that similar accelerations might eventually become possible also for individual complex probes (e.g. cosmid, YAC probes) and for genomic probes used in Comparative Genomic Hybridization (CGH; Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993; Bornfleth *et al.*, 1996).

So far, painting of chromosome territories in cell nuclei using the present protocol yielded ambiguous results. A clear territorial painting was not observed. This may turn out to be a problem of the penetration of probe molecules into the nucleus, of their diffusion characteristics there, and of the target accessibility. It might be overcome by an appropriate modification of the "Non-Formamide Protocol".

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