



Towards unlimited colors for fluorescence *in-situ* hybridization (FISH)

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Abstract

We describe a FISH protocol that allows rehybridization of complex DNA probes up to four times to the same specimen. This strategy, which we termed ReFISH, opens a wide range of new applications to conventional band pass filter epifluorescence microscopy. These include M-FISH karyotyping and cross-species color banding that emulate multiplex probe sets labeled with up to 12 fluorochromes in sequential hybridizations to the same specimen. We designed a human 24-color karyotyping probe set in combination with a 29-color cross-species color banding probe set using gibbon painting probes. Applying the ReFISH principle, 53 painting probes on individual metaphases were discriminated. This allowed simultaneous screening for inter- and intrachromosomal rearrangements on normal human diploid cells, a HeLa derived cell line, and highly rearranged gibbon chromosomes. Furthermore, the present ReFISH experiments successfully combine 24-color FISH with laser scanning confocal microscopy to study the 3D organization of all 46 human chromosome territories in individual interphase cell nuclei.

Introduction

Fluorescence *in-situ* hybridization (FISH), in particular chromosome painting with human chromosome-specific probes has contributed significantly to progress in human karyotype analysis (Ferguson-Smith 1997, Luke & Shepelsky 1998, Raap 1998, Ried *et al.* 1998). Recently, a number of different strategies have been introduced to delineate all 24 human chromosomes in different colors in a single FISH experiment, termed 24-color FISH karyotyping or genome painting. Most of these strategies are based on labeling of DNA probes in Boolean combinations with five or more different fluorochromes (Schröck

et al. 1996, Speicher *et al.* 1996, Roberts *et al.* 1999). These techniques require a highly specialized microscopic setup and imaging software using either various narrow band pass fluorescence filters (M-FISH) or an interferometer (Spectral Karyotyping, SKY).

Painting of entire karyotypes is also accomplished with four fluorochromes using a technique called ‘combined binary ratio labeling’ (COBRA, Tanke *et al.* 1999). When labeled with five fluorochromes the latter approach potentially expands the maximum number of distinguishable probes up to 56, compared with 31 by conventional combinatorial labeling. Alternatively, ‘color changing karyotyping’ (CCK; Henegariu *et al.*

1999) requires only three fluorescent dyes in order to distinguish up to 41 different probes and standard microscope equipment. This procedure is based on combinatorial probe labeling and sequential signal detection of direct and indirect labeled probes taking advantage of different signal intensities of these labels.

Naturally, any strategy that employs human chromosome specific painting probes, only allows the identification of whole chromosomes and is limited to the analysis of translocations. Without further differentiation of chromosomal subregions, intrachromosomal rearrangements escape analysis. Subchromosomal differentiation can be provided by probes derived by microdissection of chromosome segments or by so-called 'chromosome bar codes' – banding patterns generated by multicolor FISH. 'Bar codes' are designed by differential labeling and pooling of appropriate subregional DNA probes and can delineate single chromosomes (Ried *et al.* 1992, Chudoba *et al.* 1999) and subsets of chromosomes, but also the entire human chromosome complement in a single experiment (Müller *et al.* 1997, 1998). One of these strategies for the simultaneous differentiation of the entire human karyotype with 'bar codes' has been proposed, termed 'cross-species color segmenting' or Rx-FISH. It is based on a three fluorochrome/7 color 'bar code' composed of gibbon chromosome-specific painting probes (Müller *et al.* 1998).

These various multicolor approaches illustrate the demand for hybridization of increasingly complex probe sets, which is, however, limited by the number of discernable fluorochromes and fluorescent filters available. This limitation makes rehybridization of different probes on the same specimen highly attractive. Various different protocols for sequential hybridizations to the same specimen have been published (Heslop-Harrison *et al.* 1992, Epstein *et al.* 1995, Spathas *et al.* 1994, Wang *et al.* 1995, Zhen *et al.* 1998, Ye *et al.* 2001). The protocols show that it is possible to reprobe plant and animal chromosome and interphase nuclei preparations several times without significant loss of quality of the hybridization signal and cell/chromosome morphology. Thus, re-probing could also be used with highly complex probe compositions. Here, we use sequential

hybridizations as an alternative approach to multiplex FISH, which we refer to as ReFISH. We designed a 24-color karyotyping probe and a 29-color 'chromosome bar code' probe based on probe labeling in Boolean combinations. We combined both probe sets to be used in several rehybridizations that allowed both simple identification and subregional definition of chromosomes. These probes were used to simultaneously study inter- and intrachromosomal rearrangements in a human cancer cell line, to delineate the evolutionary chromosome reshuffling between gibbon species and to identify all 46 different human chromosome territories in 3D-preserved interphase nuclei.

Materials and methods

Cell samples and culture, metaphase and cell nuclei preparation

Metaphase spreads for *in-situ* hybridization experiments were prepared from PHA stimulated peripheral lymphocytes of a normal human male, a HeLa contaminant cell line (American Type Culture Collection no. CCL-6) and a lymphoblastoid gibbon (*Hylobates lar*) cell line according to standard procedures. The gibbon cell line, HY35, was the same as described by Jauch *et al.* (1992). Cell line CCL-6 was originally thought to be derived from normal embryonic intestinal tissue; however, further analysis indicated a HeLa cell contamination instead (Lavappa 1978). Preparation of 3D-preserved interphase nuclei from karyotypically normal human male fibroblast cells was performed according to Solovei *et al.* (in press).

Multiplex probe composition

Multiplex probes for human 24-color FISH karyotyping and 29-color cross-species color banding were designed by combinatorial probe labeling of DOP-PCR (Telenius *et al.* 1992) amplified human or gibbon chromosome-specific painting probes (Table 1). Gibbon painting probes have been previously described in Müller *et al.* (1998). For the composition of multiplex probes, the protocol introduced by Roberts *et al.* (1999)

Table 1. Combinatorial probe labeling scheme and color assignment (r = red, g = green, b = blue) for (a) human 24-color karyotyping, and (b) gibbon chromosome-specific painting probes used in cross-species color banding experiments. The majority of painting probes in (b) were derived from *Hylobates concolor*. 1b and 22b represent *H. concolor* polymorphic chromosome forms. Probes 5A, 5B, 6A, 6B, 9A and 9B are derived from the Siamang (*H. syndactylus*) and are equivalent to *H. concolor* chromosome arm-specific probes.

a)

Human 24 colour karyotyping																									
subset	pool	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
H1	1.1r				X			X	X	X				X			X			X	X		X		
	1.2g							X	X		X				X			X		X		X		X	
	1.3b											X				X			X		X	X			X
H2	2.1r	X		X		X			X	X	X	X											X		
	2.2g	X	X					X					X	X	X	X							X		
	2.3b			X	X		X						X					X	X						

b)

Gibbon cross species 29 colour banding																														
subset	pool	1b	2	3	4	5A	5B	6A	6B	7	8	9A	9B	10	11	12	13	14	15	16	17	18	19	20	21	22b	23	24	25	X
G1	1.1r							X	X	X	X	X	X	X			X			X	X		X		X	X		X		
	1.2g	X		X							X	X	X	X	X			X			X	X	X	X			X	X	X	
G2	2.1r		X		X	X	X		X					X				X		X	X			X		X	X	X	X	
	2.2g			X	X	X		X				X				X		X	X	X	X	X	X	X	X	X				
	2.3b	X				X	X				X					X	X	X		X		X	X	X	X			X	X	X

was adapted. Briefly, depending on the respective chromosome size and paint quality, 100–200 ng DOP-PCR pre-amplified painting probe of each probe pool member was mixed. Subsequently, the whole pool was re-amplified by DOP-PCR, using 150 ng template DNA. In a further round of DOP-PCR, each probe pool was labeled with fluorochrome or hapten-conjugated dUTPs. In order to be hybridized simultaneously, each 2.5 µg of the appropriate probe pools were mixed (for example G2.1, G2.2 and G2.3, to form probe subset G2) with 10 µg human cot-1 DNA, ethanol precipitated and resuspended in hybridization buffer (50% formamide, 1× SSC, 10% dextrane sulphate). When performing four consecutive hybridizations with both gibbon and human probe sets, the labeling scheme and hybridization sequence was as followed: hybridization 1 (G2.1D, G2.2T, G2.3B), hybridization 2 (H2.1B, H2.2D, H2.3T), hybridization 3 (H1.2T, H1.3SG) and hybridization 4 (G1.1D, G1.2B, H1.1T). (B = Biotin-dUTP, and D = Digoxigenin-dUTP,

Roche, T = Tamra-dUTP, Applied Biosystems/Perkin Elmer and SG = Spectrum Green-dUTP, Vysis) (Table 1).

(Re-)hybridization in situ and probe detection

When a chromosome specimen was hybridized the first time, DNA probes were denatured at 70°C for 7 min and pre-annealed by incubation at 37°C for 30 min. The microscope slides were denatured in 70% formamide/2× SSC at 72°C for 1 min 30 s (metaphase spreads) or 3 min (3D preserved cell nuclei preparations). The hybridization was carried out for 48 h, followed by serial washings of 2× 5 min in 50% formamide/2× SSC, 45°C, 2× 5 min 2× SSC, 45°C and 1× 5 min 0.1× SSC, 60°C. Biotinylated DNA probes were detected by Avidin-Cy5 (Amersham), digoxigenin-labeled probes by sheep anti-digoxigenin FITC conjugated antibody (Roche).

When two or more sequential hybridizations were performed, after each hybridization,

coverslip and antifading solution were removed by soaking the slide in $4 \times \text{SSC}/0.2\%$ Tween and further washing for 60 min at room temperature, followed by serial ethanol dehydration (70%, 90%, 100%). The slide was then fixed in methanol/acetic acid (3/1 v/v, 30 min, room temperature) and incubated overnight at 37°C in a dry oven. Before rehybridization of 3D preserved cells, the slide was incubated in 3.7% p-formaldehyde (20 min), $1 \times \text{PBS}$ (3×5 min) and $2 \times \text{SSC}$ (10 min), followed by overnight incubation in 50% formamide/ $2 \times \text{SSC}$. Each step was performed at room temperature.

Hybridized probe was removed during subsequent denaturation of the slide. For each round of rehybridization, the slide denaturation time was increased by 30 s (metaphase preparations) or 2 min (3D preserved cell preparations). The DNA probe was pretreated and hybridized as described above. When more than two consecutive FISH rounds were performed, only directly fluorochrome-labeled probes (Tamra-dUTP and SpectrumGreen-dUTP) were used for the third round since hapten-labeled probes were not sufficiently removed by the previous denaturation step. According to this procedure, it was possible to use hapten labeled probes again in a fourth FISH round.

Microscopic setup and image analysis

After each FISH round, hybridization images were acquired together with the coordinates of the cell on the slide with an X/Y motorized stage. Metaphases were visualized with a cooled CCD camera (Photometrics NU200 equipped with a KAF1400 chip), coupled to a Zeiss Axiophot microscope. CCD camera and stage were controlled by SmartCapture Viewpoint software (DigitalScientific, Cambridge, UK). 3D preserved cell preparations were analyzed by a three-channel

laser scanning confocal microscope (Zeiss LSM 410). Light optical serial sections (200 nm) were recorded as 8-bit gray-scale images (256×256 pixels). Image merging, pseudo coloring and generation of 3D maximum intensity projections was performed by Imaris software v. 3.0.2 (bitplane AG).

Results and discussion

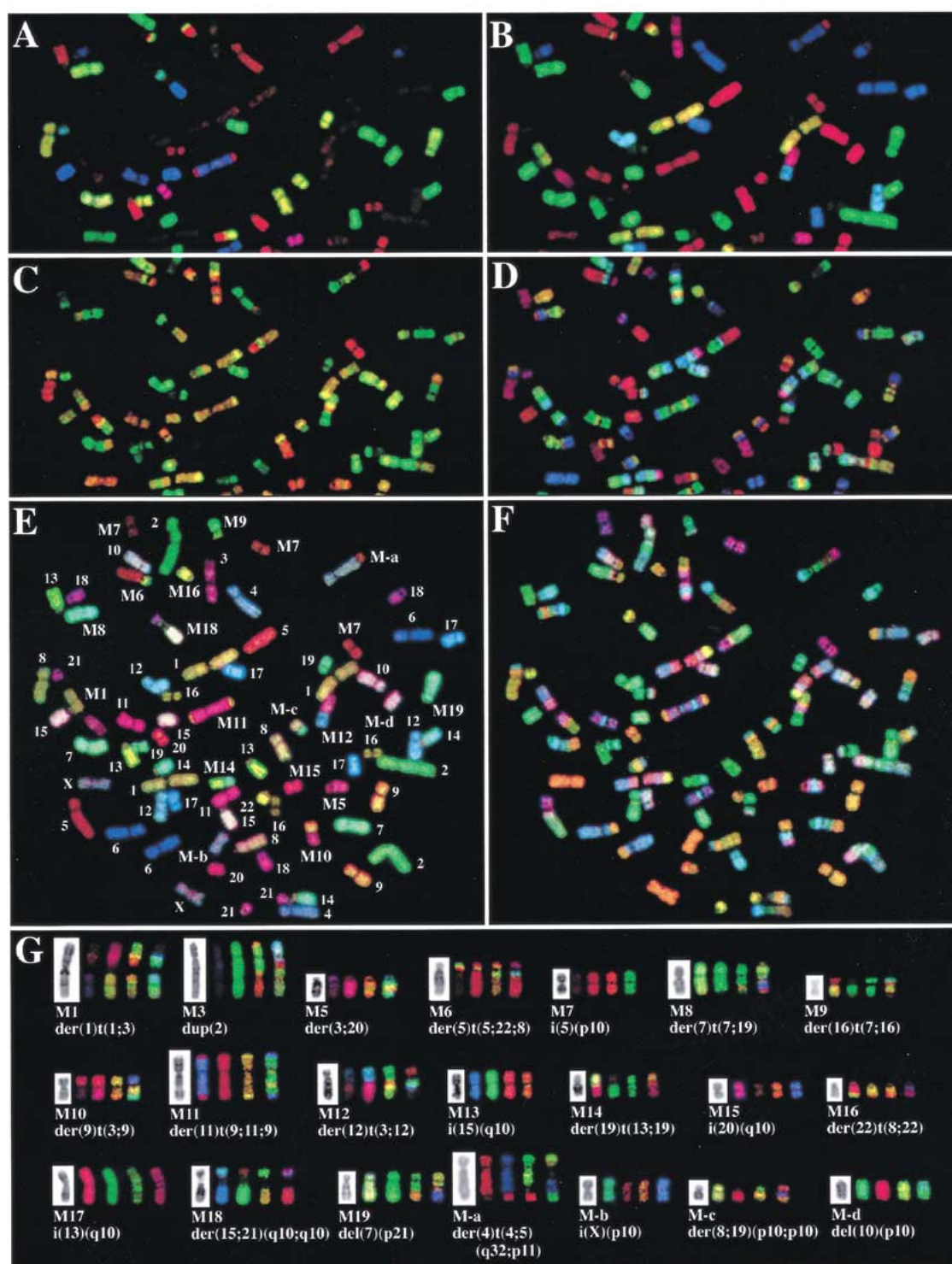
Hybridization on metaphase chromosomes derived from human lymphocytes

Human and gibbon subsets, H1, H2, G1 and G2, were sequentially hybridized to metaphase preparations from human lymphocytes as chromosomal template (data not shown). After four rounds of hybridizations, approximately 90% (15/17) of metaphases were intact and could be fully analyzed. Mapping position of each gibbon probe on human chromosomal regions was the same as previously published (Müller et al. 1998) except for *H. concolor* chromosomes 4 and 18, for which additional homologous regions to human 8p22-pter (HCO 4) and 4q26 (HCO 18) were revealed. In contrast to previous 'cross-species color banding' experiments (Müller et al. 1998), the combination of both human and gibbon probe sets defined each subchromosomal segment by a unique color code. No color redundancy in the 'bar code pattern' was observed anymore; thus in every case a precise identification of chromosomal material involved in translocations was possible.

Analysis of HeLa contaminant cell line CCL-6

A HeLa cervical carcinoma cell line contaminated cell line CCL-6 was analyzed with both 24-color chromosome painting and cross-species color banding (Figure 1). Fifteen cells were fully

Figure 1. Representative metaphase of the HeLa contaminant cell line CCL-6 after four rounds of ReFISH for human 24-color karyotyping and 29-color cross-species color banding. (A) Human probe subset H1, and (B) subset H2, (C) gibbon probe subset G1 and (D) subset G2. Probes present in more than one pool of a subset showed a defined mixed color of the RGB spectrum (see Table 1 for probe composition and color assignment). (E) False color display after merging of all 6 gray-scale image planes derived from the human 24-color probe subsets, H1 and H2, with assignment of normal and marker chromosomes. (F) Display of the gibbon 29 color probe subsets, G1 and G2. (G) Summary of marker chromosomes identified in cell line HeLa CCL-6. Each chromosome is shown with the inverted DAPI counter stain and all four probe subsets (left to right).



analyzed and compared with HeLa CCL-2, which has been previously analyzed by Spectral Karyotyping (SKY) by Macville *et al.* (1999).

The majority of clonally aberrant HeLa markers M1–M20 described for HeLa CCL-2 were also observed in the cell line CCL-6 (Figure 1E). Markers M21–M31 that were previously identified in a fraction of CCL-2 cells were not observed in CCL-6. Neither could markers M2, M4 and M20 be observed; they were not present in any cell. To obtain further confirmation about the absence of markers M2 (der(1;9)(p10;q10)), M4 der(3;5)(p10;q10) and M20 (der(7)t(3;7)(p21;p21)), we performed two-color hybridizations with the appropriate paint combinations (chromosomes 1/9, 3/5 and 3/7, respectively). We could, however, not identify these markers in cell line CCL-6 in any of the 50 scored cells (data not shown). Two further markers were found only in a fraction of cells (M13 in 40% and M17 in 20% of cells analyzed). We also identified four clonal markers in CCL-6 that were not described for HeLa CCL-2, termed M-a to M-d. The hybridization pattern of all marker chromosomes observed in CCL-6 is summarized in Figure 1G.

Differences in various marker chromosomes between CCL-2 and CCL-6 were most probably due to independent gains or losses during propagation of the two cell lines. It is highly unlikely that markers M2, M4 and M20 were not detected by ReFISH because of insufficient resolution or sensitivity of the approach since they are translocation products involving large chromosomal fragments and were also not found by conventional dual-color FISH control experiments. Further analysis of different HeLa derivative cell lines may be necessary to reconstruct a 'consensus' HeLa karyotype and to distinguish cell culture artifacts from originally present and potentially significant chromosome rearrangements for cervical carcinomas.

Comparative chromosome analysis of human and gibbon chromosomes

The karyotypes of gibbons (Lesser Apes, Primates) not only differ from their human homologs but also between gibbon species by extensive chromosome reshuffling. Up to now, chromosomal

phylogenies could not be established using human painting probes alone (Jauch *et al.* 1992, Koehler *et al.* 1995a, 1995b, Yu *et al.* 1997) since ancestral vs. derived chromosome forms could not be established. These data can be obtained when additionally analyzing chromosome rearrangements between gibbon species, in particular when they are compared with a putative ancestral hominoid karyotype (Müller & Wienberg 2001). Further, a combined human and gibbon probe set makes it possible to directly distinguish between similar and identical breakpoints in different gibbon species. We analyzed the Lar gibbon (*Hylobates lar*) in four consecutive hybridizations with probe sets H1, H2, G1, G2 (Table 1, Figure 2A, B).

Previous hybridization results using human paints (Jauch *et al.* 1992) were confirmed with the exception of minute signals on Lar gibbon chromosomes 5 and 12, where additional human homologous chromosome 11 and 8 material, respectively, was detected. The homologous probes 4 and 8 of the Concolor gibbon which were previously mapped to human chromosome bands 11q12-13.1 and 8p11-22 (Müller *et al.* 1998) further supported this finding in *H. lar*. The comparative chromosome map between human, *H. concolor* and *H. lar* is summarized in Figure 3.

Recently, we suggested that the majority of gibbon chromosome forms can be derived from the ancestral hominoid karyotype by fissions and/or translocations without further intra-chromosomal rearrangements (Müller & Wienberg 2001). For example, the human chromosome 7 homolog shows the same subchromosomal organization in *H. lar* and the putative ancestral hominoid, and therefore may have been also present in the ancestral hylobatid. In contrast, the human chromosome 7 homologs in *H. concolor* are clearly derived by further translocations. Correspondingly, ancestral gibbon chromosome forms may have been conserved in *H. lar* chromosomes 2p (HSA 10q), 3p (HSA 6q), 4q (HSA 13), 13p (HSA 17q), 13q (HSA 9q), 17 (HSA 14), 19 (HSA 1q25-qter), 20 (HSA 6p) and 21 (HSA 20). Further ancestral gibbon chromosome forms are evident in *H. concolor* chromosomes 16 (HSA 8q), 21 (HSA 3p24-pter; p14-q22), 23 (HSA 12p), 24 (HSA 1p34.2-pter)

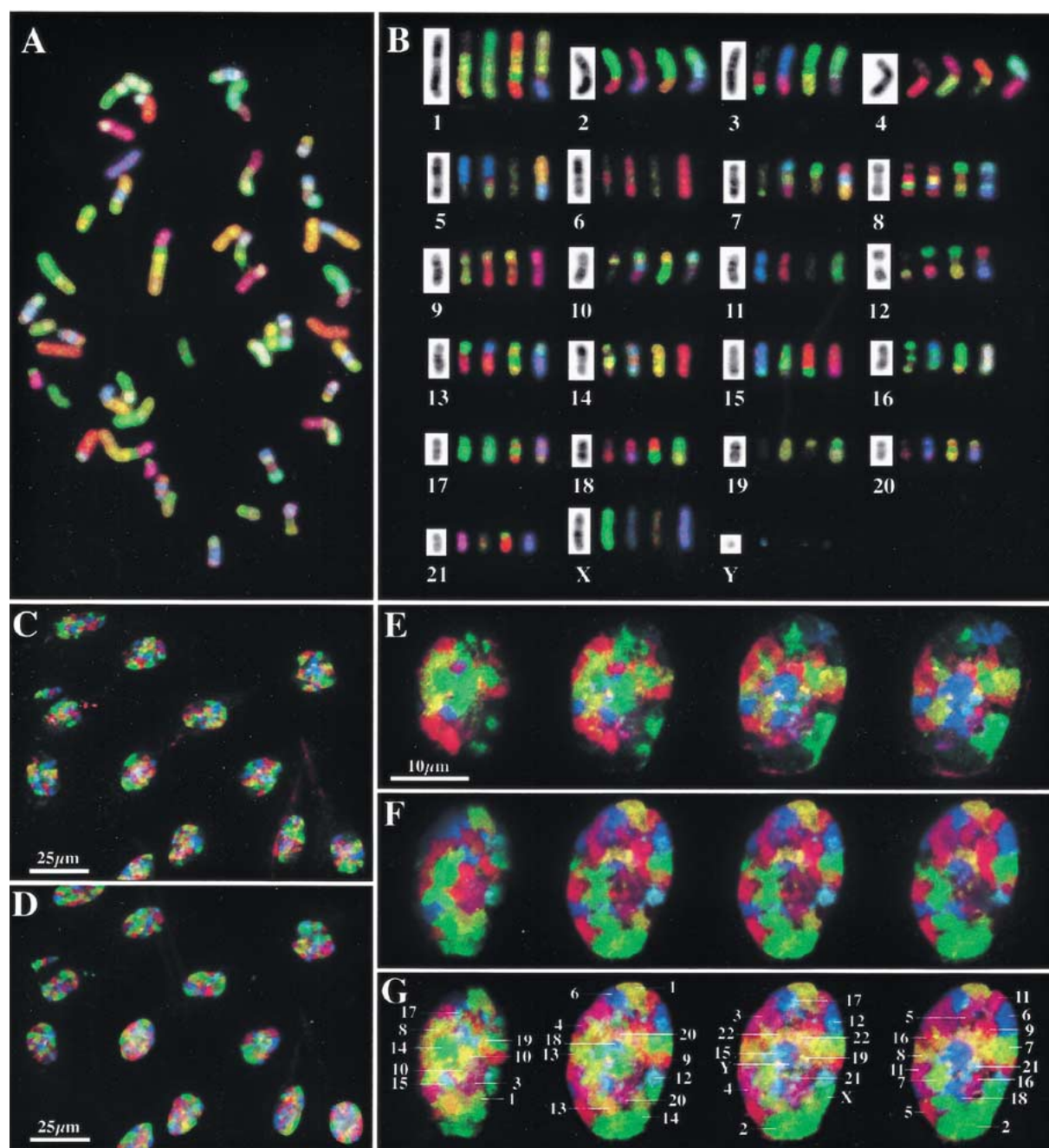


Figure 2. (A) and (B) Differentiation of a *Hylobates lar* (gibbon) metaphase after four rounds of ReFISH with human and gibbon probe subsets (see Table 1 for probe composition). (A) False color display of the gibbon 29-color probe set (combined subsets G1 and G2) illustrates the drastic evolutionary karyotype changes between different gibbon species. The cell line HY35 analyzed is partially triploid. (B) Half karyotype of the same *H. lar* metaphase. Each chromosome is shown with the inverted DAPI counter stain and all four probe subsets (left to right). (C–G) Human 24-color chromosome painting to 3D preserved human interphase nuclei by ReFISH: RGB displays of (C/E) human probe subset H1, and (D/F) subset H2. (C) and (D) show low magnification confocal midsection images and provide an overview of the sequential hybridization efficiency in 3D preserved cell preparations. (E) and (F) Successive 3D maximum intensity projections of an individual nucleus (sections 8–40 of 48). (G) Overlay of (E) and (F) with manual classification of all 46 individual chromosome territories.

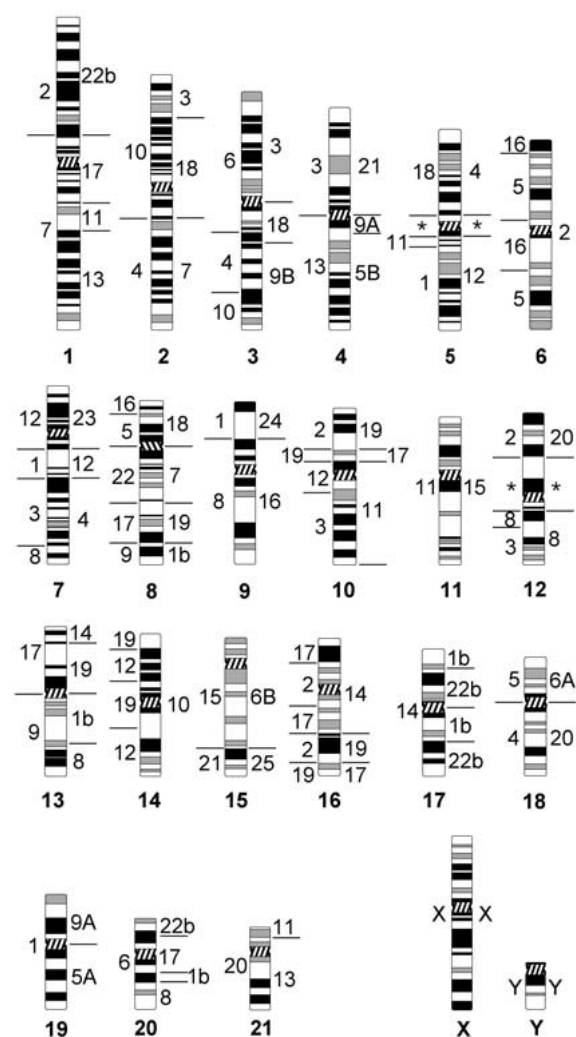


Figure 3. Idiogram based on the G-banding karyotype of the gibbon *H. lar*, together with the assignment of all human (left) and *H. concolor* (right) homologous chromosomal regions. Regions marked with asterisks were not hybridized. Chromosomal regions homologous to *H. concolor* 5A, 5B, 6A, 6B, 9A and 9B were detected by Siamang painting probes which show homology to entire arms of *H. concolor* chromosome 5, 6 and 9.

and 25 (HSA 21). The present data and those presented by Nie *et al.* (2001), who directly compared *H. concolor* and *H. hoolock* chromosomes, are the first steps towards a reconstruction of the ancestral gibbon karyotype. The final picture will arise when the karyotype of the Siamang (*H. syndactylus*) is compared with other gibbons. This work is in progress.

Analysis of all chromosome territories in human 3D preserved interphase nuclei

The present ReFISH experiments successfully combined human 24-color FISH with laser scanning confocal microscopy to study the 3D organization of all 46 chromosome territories in individual interphase cell nuclei. Probe subsets, H1 and H2, were sequentially hybridized to 3D preserved human fibroblast interphase nuclei (Figure 2C–G). For each cell, confocal serial sections were recorded from both hybridizations. Six different grayscale image planes were obtained of each section and merged to binary RGB displays. For further analysis, these RGB images were transformed to serial 3D maximum intensity projections, equivalent to approximately 1.5 μm each (7–8 consecutive confocal sections).

Ten cells were analyzed by visual classification for each of the serial 3D projections. This led to the following observations: after adaptation of the cell fixation protocol to the requirements of complete probe removal and optimal preservation of nuclear morphology, a reproducible hybridization pattern was observed in the majority of cells. It was prerequisite that cells were not grown to confluence, since in confluent cells an excessively dense extracellular matrix prevented probe or antibodies from efficiently penetrating the nucleus. As revealed by the number of obtained optical serial sections in the sequential hybridizations, a reduction of the interphase nucleus size of approximately 10% in the Z-axis and of less than 5% in diameter was noticed after the second hybridization, although neither nuclear morphology nor the boundary shape of chromosome territories changed. Chromosome identification presented no difficulties in the nuclear periphery, but in some instances in the nuclear interior, in particular in areas where nucleolar organizing region (NOR)-bearing chromosomes were located, resulting in an overall classification efficiency of over 90% of chromosome territories per nucleus.

Up to now, the study of higher-order organization of chromosome territories in interphase is still hampered by the fact that laser scanning confocal microscopes generally only discriminate up to three different fluorochromes. This technical limitation may now be overcome by the ReFISH

approach, provided that protocols for metaphase rehybridization are adapted to special requirements of interphase FISH. In particular, the preservation of nuclear morphology is important for the correct classification of chromosome territories. This is true for diagnostic applications on methanol/acetic acid fixed cells, but essential in the study of nuclear architecture, in which 3D preserved nuclei are investigated. Furthermore, since nuclear morphology as well as the boundary shapes of individual chromosome territories could be well preserved, a minor reduction of the overall nuclear volume appears to present no significant obstacle to future systematic studies of nuclear architecture by ReFISH.

In conclusion, the experiments presented in this study demonstrate that the ReFISH approach is applicable for complex multicolor probes in a variety of cytogenetic fields, both in the study of metaphase chromosomes and interphase nuclei. The approach demonstrates the possibility to combine more than one multiplex probe, dedicated to the analysis of different spectra of chromosomal aberrations like inter- and intrachromosomal aberrations. This strategy could also be extended to the combined application of chromosome painting and subtelomere FISH for the detection of cryptic rearrangements.

Alternatively, the design of even more complex multicolor probe sets can be imagined. Recently, M-FISH experiments have been described using seven different fluorochromes in a single experiment (Saracoglu *et al.* 2001). ReFISH with four hybridizations and seven fluors per sequential hybridization theoretically expands the limit of probes to be differentiated simultaneously up to 2^{28} , provided all probes would be labeled in Boolean combinations ($2^{N \times M}$; where N = number of fluors, M = number of sequential hybridizations). The spatial resolution of metaphase chromosomes, however, would already be exceeded by only two sequential hybridizations with seven fluors each (2^{14} signals), which is superior to any described chromosome banding pattern.

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