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Multiplex-FISH for Pre- and Postnatal Diagnostic Applications

Sabine Uhrig,[1] Simone Schuffenhauer,[2] Christine Fauth,[1] Antje Wirtz,[2] Cornelia Daumer-Haas,[3] Can Apacik,[4] Monika Cohen,[4] Jutta Müller-Navia,[5] Thomas Cremer,[1] Jan Murken,[2] and Michael R. Speicher[1]

[1]Institut für Anthropologie und Humangenetik and [2]Abteilung für medizinische Genetik der Kinderklinik, Ludwig-Maximilians-Universität München, [3]Frauenärzte und medizinische Genetik, and [4]Kinderzentrum München, Munich; and [5]Institut für klinische Genetik Mainz, Mainz, Germany

Summary

For >3 decades, Giemsa banding of metaphase chromosomes has been the standard karyotypic analysis for pre- and postnatal diagnostic applications. However, marker chromosomes or structural abnormalities are often encountered that cannot be deciphered by G-banding alone. Here we describe the use of multiplex-FISH (M-FISH), which allows the visualization of the 22 human autosomes and the 2 sex chromosomes, in 24 different colors. By M-FISH, the euchromatin in marker chromosomes could be readily identified. In cases of structural abnormalities, M-FISH identified translocations and insertions or demonstrated that the rearranged chromosome did not contain DNA material from another chromosome. In these cases, deleted or duplicated regions were discerned either by chromosome-specific multicolor bar codes or by comparative genomic hybridization. In addition, M-FISH was able to identify cryptic abnormalities in patients with a normal G-karyotype. In summary, M-FISH is a reliable tool for diagnostic applications, and results can be obtained in ≤24 h. When M-FISH is combined with G-banding analysis, maximum cytogenetic information is provided.

Introduction

Since the discovery, by Zech and Caspersson (Caspersson et al. 1968, 1970), that appropriate staining results in a banded appearance of chromosomes, various methods for banding of metaphase chromosomes have been used as standard techniques in pre- and postnatal diagnostic applications. Giemsa bands obtained by digestion of the chromosomes by the proteolytic enzyme trypsin (GTG-bands) are the bands most widely used for routine chromosome analysis in clinical laboratories. However, GTG-banding can achieve a resolution to only the single-band level—that is, ~5–10 million bp. Thus, it is not surprising that another option for karyotype analysis, FISH, has become very popular in diagnostic applications. In the past, only a limited number of DNA probes could be hybridized simultaneously. As a consequence, selection of the probe was crucial, with some prior knowledge necessary for the selection of the appropriate probe (e.g., see Jauch et al. 1990; for review, see Carter 1996). More recently, new technologies allowing the identification of the two dozen different human chromosomes—22 autosomes and the X and Y sex chromosomes—each with a distinct color, have extended traditional FISH applications to screening of the entire genome, for both numerical and structural abnormalities, in a single hybridization (Speicher et al. 1996; Schröck et al. 1996; for review, see Lichter 1997). The use of multiplex-FISH (M-FISH; Speicher et al. 1996) in pre- and postnatal diagnostic situations is described here. M-FISH exploits the combinatorial probe-labeling strategy, the simplest means for generation of color combinations far in excess of the number of spectrally resolvable fluorophores. Only five fluorophores are needed to distinguish the 24 human chromosomes. By hybridization of sets of chromosome-specific DNA probes, each labeled with a different combination of fluorescent dyes, to metaphase chromosome spreads, it is possible to ascribe to each chromosome a unique spectral signature or identifier tag. Recent advances in the M-FISH technology include new labeling strategies; automation of the image-capturing procedures, by use of a motorized epifluorescence microscope; and novel multichannel image-analysis methods (Eils et al. 1998; Bolzer et al. 1999). These improvements allow M-FISH results to be available in <24 h.

We have used M-FISH in 121 diagnostic applications on slides provided from 18 different laboratories, and we summarize here some of the most interesting cases.

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Address for correspondence and reprints: Dr. Michael R. Speicher, Institut für Anthropologie und Humangenetik, LMU München, Goethestrasse 31, D-80336 München, Germany. E-mail: speicher@fish.med.uni-muenchen.de

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Table 1

Summary of Patients Referred from Various Laboratories, for M-FISH Analysis

Patient (Indication for Chromosome Analysis)	Karyotype, Based on		
	G-Banding	M-FISH	G-Banding + M-FISH + Additional FISH Experiments
1 (Prenatal diagnosis, advanced maternal age)	47,XX,+mar	47,XX,+psu idic(15)(q11~13)[a]	47,XX,+psu idic(15)(q12 or q13)
2 (Prenatal diagnosis, advanced maternal age)	47,XX,+mar	47,XX,+psu idic(15)(q11~13)[a]	47,XX,+psu idic(15)(q11)
3 (Cat-eye syndrome)	47,XX,+mar	47,XX,+psu idic(22)(q?1)[a]	47,XX,+psu idic(22)(q11.21)
4 (Turner syndrome)	45,X/46,X,+mar	45,X/46,X,psu idic(Y)(q11.2)[a]	Not done
5 (Turner syndrome)	45,X/46,X,+mar	45,X/46,X,psu idic(Y)(q11.2)[a]	Not done
6 (Prenatal diagnosis, advanced maternal age)	47,XX,+mar	47,XX,+der(?)t(?;18)(?;?)	47,XX,+der(21)t(18;21)(p11.2;q11.1)
7 (Prenatal diagnosis, advanced maternal age)	47,XX,+mar	47,XX,+der(?)(h)	47,XY,+der(7)(:p11→q11.1:)
8 (Prenatal diagnosis, advanced maternal age)	47,XY,+mar	47,XY,+der(?)(h)	47,XY,+i(14 or 22)(p10)
9 (Infertility work-up)	47,XY,+mar	47,XY,+der(?)(h)	Not done
10 (Prenatal diagnosis, history of recurrent abortions)	46,XX,t(1;21)(p36.3;q22.1)	46,XX,t(1;21)(p36.3;q22.3)	Not done
11 (Wolf-Hirschhorn syndrome)	46,XX,der(4)(qter→p15::?)	46,XX,der(4)t(4;13)(p15;q32)	Not done
12 (Prenatal diagnosis, abnormal ultrasound)	46,XX,?del(4)(p16)	46,XX,der(4)t(4;X or 9)(p16;?)	46,XX,der(4)t(4;9)(p16.1;p23~p24)
13 (Thymic aplasia, cardiac anomaly)	46,X,add(X)(p11)	46,X,der(X)t(X;9)(p11;?)	46,X,der(X)t(X;9)(p11;q31)
14 (Mental retardation, dysmorphic features, cardiac anomalies)	46,XY,add(8)(q24)	46,XY,?ins(8;5)(q24.1;q11.2q22)	46,XY,ins(8;5)(q24.1;q11.2q22)
15 (Mental retardation, dysmorphic features)	46,XX,add(20)(p13)	46,XX,der(20)t(6;20)(?;p?)	46,XX,der(20)t(6;20)(p24;p13)
16 (Prenatal diagnosis)	46,XX,add(10)(q26)	46,XX,der(10)t(10;12)(q26;?)	46,XX,der(10)t(10;12)(q26;q24.1)
17 (Prenatal diagnosis, mother of case 18)	46,XX,t(2p;8p)	46,XX,t(2;17;8)(p23;p11.2;p12)	Not done
18 (Prenatal diagnosis, fetus of case 17)	46,XX,t(2p;8p)	46,XX,t(2;17;8)(p23;p11.2;p12)	Not done
19 (Prenatal diagnosis, advanced maternal age)	46,XX,add(4)(q33)	46,XX,add(4q)	46,XX,dup(4)(q31q35)
20 (Mental retardation, dysmorphic features)	46,XX,der(1q)	46,XX	46,XX,del(1)(q42)
21 (Mental retardation, dysmorphic features)	46,XX,add(1q42)	46,XX	46,XX,ins(1)(q42q41q32.1)
22 (Prenatal diagnosis, abnormal ultrasound)	46,XY,add(13)(p11)	46,XY,der(13)(qter→q21::p11→qter)	Not done
23 (Klinefelter syndrome)	46,XX	46,X,der(X)t(X;Y)(22.3;p11.3)	Not done
24 (Klinefelter syndrome)	46,XX	46,XX	Not done
25 (Mental retardation, dysmorphic features)	46,XX	46,XX,der(20)t(18;20)(q21;?)	46,XX,der(18)t(18;20)(q21;p11.2)
26 (Mental retardation, dysmorphic features)	46,XX	46,XX,der(1)t(1;12)(q43;?)	46,XX,der(1)t(1;12)(q43;p13)

[a] Based on M-FISH and CBG and Ag-NOR staining.

Table 2

YAC Clones Used to Generate Chromosome 1–Specific Multicolor Bar Codes

Map Position	YAC Clone	Fluor Combination
1p36.3	HTY3222	Cy3 + Cy3.5
1p34	907g1	Cy3 + Cy5
1p31.2-p31.3	753d12	Cy5
1p31.1	853g3	FITC + Cy3.5
1p13	942h6[a]	Cy3.5 + Cy5.5
1q22-q23	757a7	Cy3
1q32	773c6	Cy5.5
1q41	958e1	FITC
1q43-q44	YRM2123	Cy3.5

[a] Second hybridization sites are present at 10q23 and 22q11.2.

M-FISH was applied to determine the chromosomal origin of supernumerary marker chromosomes (SMCs) and to analyze metaphase spreads with structurally abnormal chromosomes the composition of which could not be deciphered on the basis of GTG-bands alone. In addition, we report our first findings from using M-FISH for screening of individuals who have mental retardation and dysmorphic features with no karyotypic abnormality detected by GTG-banding. Advantages and limitations of the M-FISH technology are discussed. In cases in which M-FISH was not sufficient to establish a diagnosis, other techniques—such as multicolor chromosome-specific-bar coding, reverse painting, or comparative genomic hybridization (CGH)—were used. The combination of all techniques allowed a definition of the structural abnormalities, with a superior resolution.

Material and Methods

Samples from Patients

Metaphase spreads from patients referred to clinical laboratories, for karyotypic analysis, were prepared according to standard procedures. Table 1 summarizes the indication for chromosome analysis and the specifications of karyotypes after different diagnostic procedures. This study was approved by the ethics committee of the University of Munich. Informed consent was obtained from all participants.

M-FISH

M-FISH was performed as described elsewhere (Eils et al. 1998), with minor modifications in pretreatment and denaturation of metaphase preparations and posthybridization washes. Pretreatment of slides consisting of either an RNase and a pepsin digestion (Lengauer et al. 1992) or pepsin digestion alone appeared to be a crucial determinant for a successful experiment. Depending on the amount of cytoplasm, the duration of the pepsin digestion (40 mg/ml) was in the range of 2–7 min. The slides were denatured in 70% formamide, 2 × SSC at 70°C. Denaturation time varied according to the age of the slides and ranged from 1 min 20 s, for slides prepared during the preceding 24 h, to ~2 min, for slides several weeks old. The slides were incubated for one night at 37°C. The posthybridization washes were three times, for 5 min each, with 4 × SSC/0.5% Tween 20 at 42°C and three times, for 5 min each, with 1 × SSC at 60°C. Biotin-labeled probes were detected with avidin-Cy3.5 (Amersham Pharmacia Biotech), and digoxigenin-labeled probes were detected with two layers, one of anti–digoxigenin-rabbit antibody (Sigma) and the other of anti–rabbit-Cy5.5 antibody (Amersham Pharmacia Biotech).

FISH, CGH, and Reverse Painting

In some cases, region-specific DNA probes were used to verify a result, to narrow the size of a chromosome fragment involved in a structural rearrangement, or to provide a higher-resolution map of a breakpoint. These probes included subtelomeric cosmids or plasmid artificial chromosomes (Ning et al. 1996) 160H23 (1q), dJ790J10 (8p), 489D14 (8q), 2189b6 (10p), 2136a1 (10q), 221K18 (12q), 2005a4 (20p); microdissected 9p-, 9q-, 18p-, and 18q-specific and 6pter-6p24 and 6q25.3-6qter band-specific probes (Guan et al. 1995, 1996); and CEPH-YAC 922c8 (12p13; Bray-Ward et al. 1996). Probes were labeled either by standard nick translation or by degenerate oligonucleotide–primed PCR (Telenius et al. 1992). CGH was performed according to standard protocols (e.g., see Speicher et al. 1993). Reverse painting, including microdissection and DOP-PCR were performed according to previously published protocols (Müller-Navia et al. 1995). Commercial LSI DiGeorge/VCFS (22q11.2) and LSI Prader-Willi/Angelman region (SNRPN; 15q11-q13) probes (both Vysis) were used according the manufacturer's instructions.

Chromosome 1 Bar Code

A chromosome 1–specific multicolor bar code was constructed by use of different YAC clones (table 2). Only YAC clones with a hybridization efficiency >99% were selected for the bar codes. YAC clones HTY3222 and YRM2123 are half-YACs specific for telomeric chromosome bands 1p36.3 and 1q44, respectively (Vocero-Akbani et al. 1996). In addition, several YAC clones were selected from the CEPH library (Bray-Ward et al. 1996). Their suitability for use as bar codes was checked by hybridization of two or three YACs simultaneously, in different colors, to normal metaphase spreads, in order to determine their relative position to each other and to map the exact band position at a high-resolution level. On the basis of these hybridization results, seven

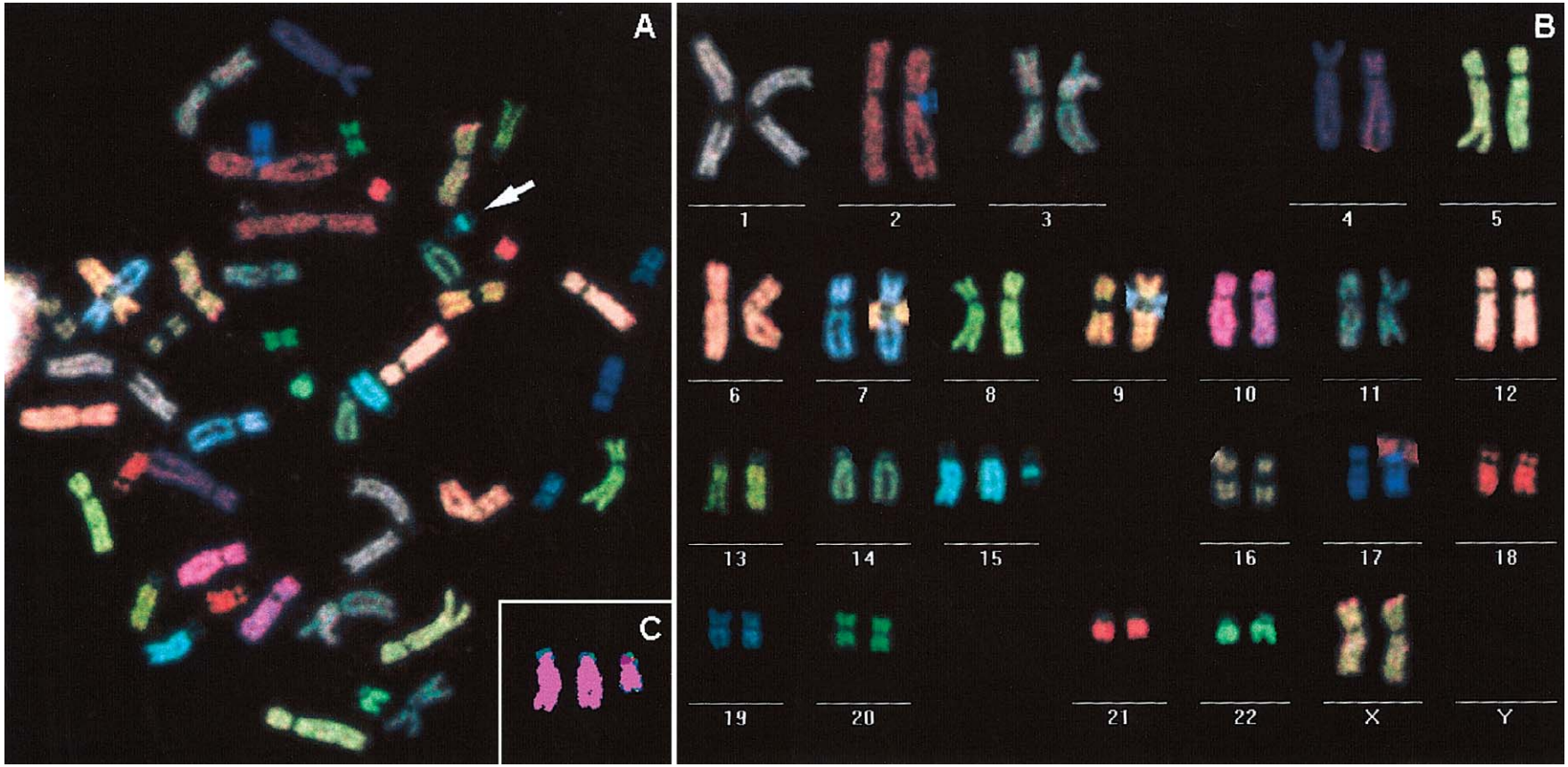
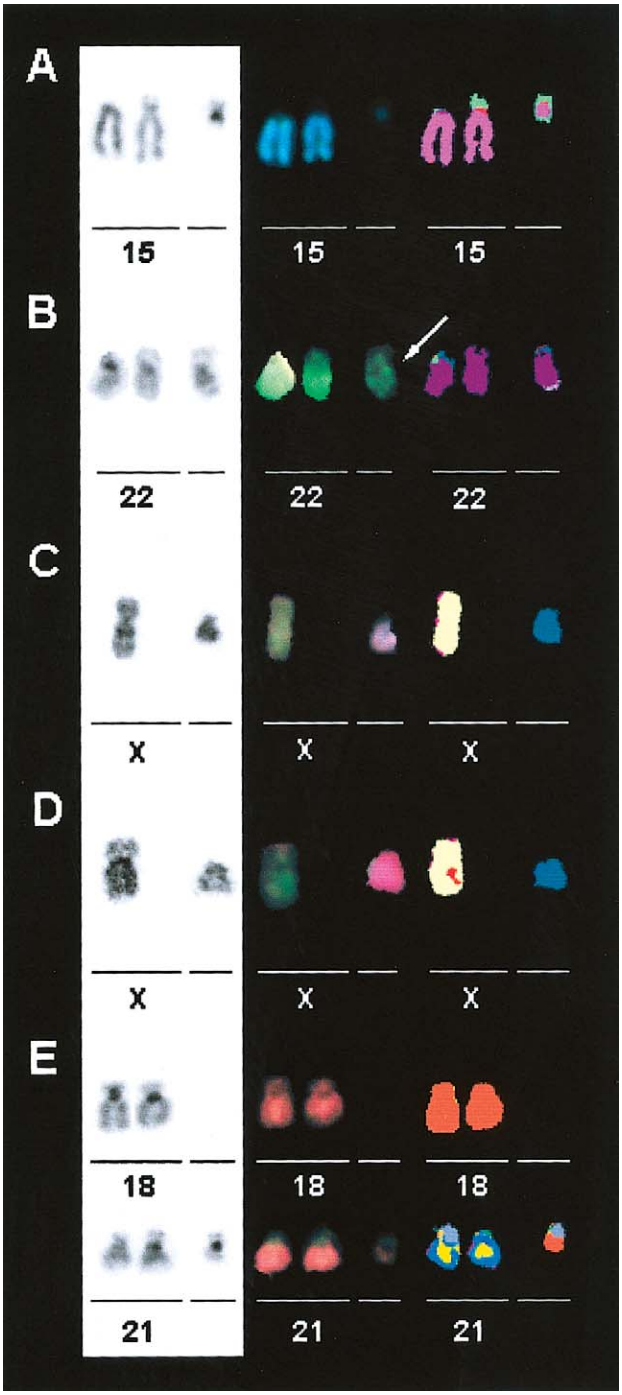


Figure 1 *A,* Metaphase spread of patient 1 after hybridization of the M-FISH probe mix, displayed in true colors. M-FISH identified euchromatin derived from chromosome 15 within an SMC (*arrow*). When results of CBG-band and Ag-NOR staining were considered together, this marker chromosome was classified as +psu idic(15)(q12 or q13). *B,* Spectral signatures of each chromosome, on the basis of which a karyogram was calculated. *C,*Two chromosomes 15 and an SMC, shown in classification colors. For discussion of the classification colors at the heterochromatic regions, see the text.

YACs were selected, in addition to the two aforementioned half-YACs. After *Alu*-PCR (Lengauer et al. 1992), YACs were labeled according to the labeling scheme in table 2.

Epifluorescence Microscopy and Image Analysis

A motorized epifluorescence microscope equipped with an eight-position filter wheel (Leica DMRXA-RF8) and a Sensys CCD camera (Photometrics [KAF 1400 chip; Kodak]) was used for image acquisition. The specification of the filter set and details about the microscope have been published elsewhere (Eils et al. 1998). Microscope and camera were controlled by Leica QFISH software (Leica Microsystems Imaging Solutions). M-FISH image processing was performed by use of the Leica MCK image-analysis package (Leica Microsystems Imaging Solutions), which is based on an adaptive region-oriented approach for spectral classification (Eils et al. 1998). M-FISH results can be displayed either as "true colors" (e.g., see fig. 1), which are the result of an overlaying of the five source images, without further image processing, or as "classification colors" (e.g., see-fig. 9), which are generated by the aforementioned classification algorithm (Eils et al. 1998). CGH experiments were evaluated by the Leica QCGH software package (Leica Microsystems Imaging Solutions). Gray-scale images with region-specific probes were overlaid without further image processing, by use of the Leica QFISH software package (Leica Microsystems Imaging Solutions). For the bar-code images, contrast enhancement, pseudocoloring, and overlaying were performed by Adobe Photoshop.



Results

Patients were referred for M-FISH analysis either because of the presence of cytogenetic abnormalities that could not be properly defined by GTG banding or because the phenotype of a patient was highly suggestive

Figure 2 Classification of SMCs in patients 2–6, by M-FISH. The first column shows the chromosomes in the inverted DAPI image, and the second and third columns show the same chromosomes in true colors and classification colors, respectively. The characterization of the SMCs is based on the M-FISH signal and on CBG-band or AgNOR staining. *A*,Second example of identification of a marker chromosome with chromosome 15 material (patient 2). Note that the signal is smaller than that in figure 1. The SMC was classified as +psu idic(15)(q11). *B*, SMC observed in a patient with cat-eye syndrome (patient 3). A small region with high fluorescence intensity and chromosome 22 spectral signature best visible in the true-color representation (second column, *arrow*) was observed; this signal indicates the presence of euchromatin. The rest of the SMC showed only very weak hybridization signals, which is consistent with the presence of heterochromatin where hybridization is suppressed. Subsequent hybridizations with region-specific probes (not shown) allowed us to classify the SMC as +psu idic(22)(q11). *C* and *D*, SMCs identified as idic(Y)(q11), observed in patients 4 (*C*) and 5 (*D*). The X chromosome is displayed as a size marker. *E*, M-FISH identification of chromosome 18 material in the SMC. Subsequent hybridizations with region-specific probes and reverse painting showed that the centromere was derived from chromosome 21 (not shown). This allowed us to classify the SMC as der(21)t(18;21)(p11.2;q11.1). Note that classification colors generated at heterochromatic regions (as in *A* and *E*) are artifacts; for details, see the text.

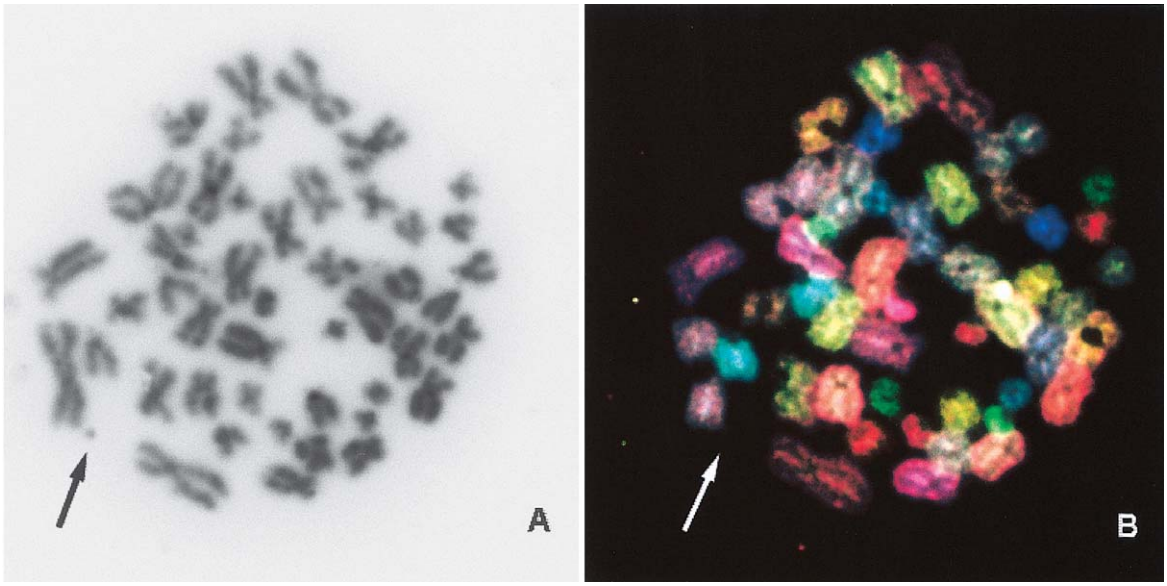


Figure 3 Analysis of metaphase spread of a direct preparation from chorionic villi with an SMC, by M-FISH (patient 8). The SMC indicated by an arrow in the inverted DAPI counterstain (*A*) shows no signal after hybridization of the M-FISH mix (*B, arrow*). The absence of a fluorescence signal suggests that the marker is composed only of heterochromatin, which was confirmed by microdissection and reverse painting (not shown).

of a chromosomal syndrome but the GTG-banded karyotype was normal.

Cytogenetic Abnormalities

The potential of M-FISH to correctly decipher small euchromatic regions in SMCs was tested. Structural aberrations that were the result of inter- or intrachromosomal rearrangements and that were observed in GTG-banding were unfolded by M-FISH. In addition, the capability of M-FISH to visualize Y-chromosome fragments in 46,XX males was determined.

SMCs.—The study was started with patients 1 and 2, in whom SMCs already had been diagnosed, by other methods, as being dicentric chromosomes 15.Figure 1 shows a metaphase spread from patient 1. A chromosome 15–specific signal is visible on the de novo SMC. Comparison with the DAPI image revealed no hybridization signal at either end of the chromosome, and a constriction indicating an active centromere was observed at one end only. FISH with the SNRPN probe demonstrated the presence of two copies of the Prader-Willi/Angelman region in this marker chromosome (data not shown). Thus, the karyotype was defined as 47,XX,+psu idic(15)(q12 or q13). In patient 2, the dicentric chromosome was smaller, but M-FISH could still correctly identify the chromosome 15 material in the SMC (fig. 2*A* and table 1). The SNRPN probe showed no hybridization signal on this SMC (data not shown), allowing us to establish the karyotype as 47,XX,+psu idic(15)(q11). As in all FISH applications performed under suppression conditions, heterochromatic regions cannot be evaluated. This is exemplified on the p arms of the normal chromosomes 15 and on the distal ends

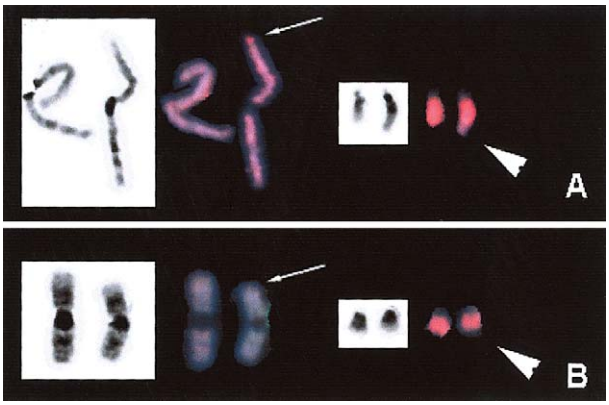


Figure 4 M-FISH analysis of a balanced translocation classified as t(1;21)(p36.3;q22.1) by GTG-banding. This example illustrates the significance that chromosome condensation has for the detection of subtle alterations. The translocation can readily be seen on long chromosomes, as shown in true colors (*A*); however, the detection becomes more difficult with increasing condensation (*B*). The arrow indicates the site where chromosome 21 material is translocated to chromosome 1, and the arrowhead indicates, vice versa, the site where chromosome 1 material is translocated to chromosome 21. The M-FISH analysis refined the breakpoint on chromosome 21, to band 21q22.3 (for details, see the text).

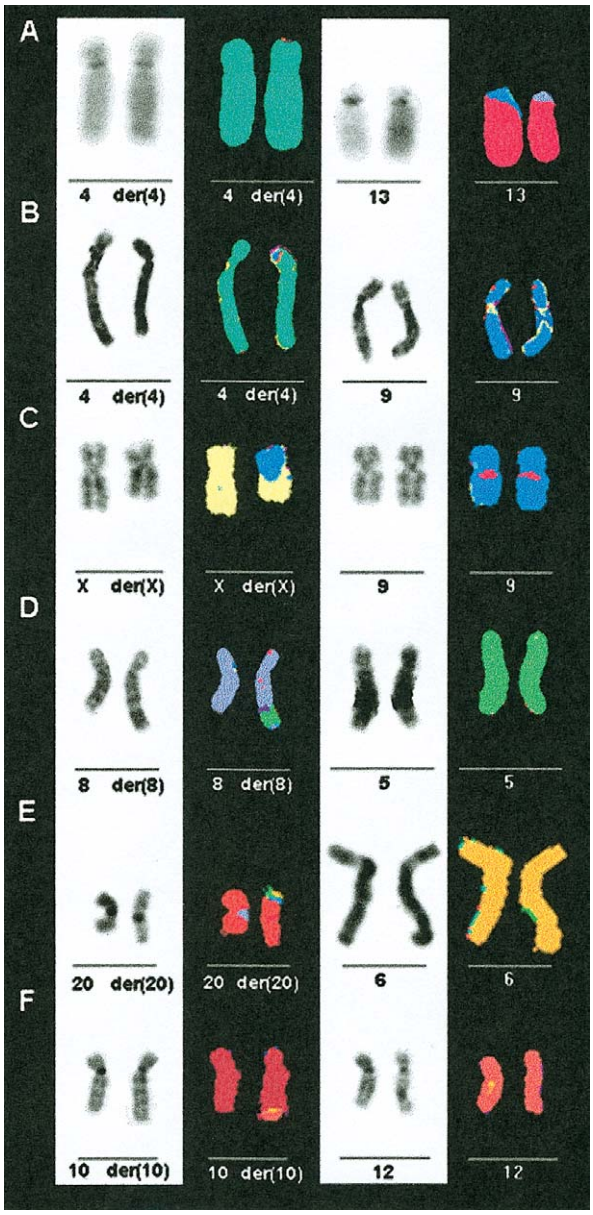


Figure 5 Summary of unbalanced rearrangements classified by M-FISH. None of these structural rearrangements was classified correctly by GTG-banding alone. The first and third columns show the chromosomes as inverted DAPI images, and the second and fourth columns show the respective chromosomes in classification colors: the derivative chromosomes with their normal homologues are shown in the first two columns; the third and fourth columns display the corresponding normal chromosome pair of the translocated or inserted chromosome material. *A,* der(4)t(4;13)(p15;q32) observed in patient 11. *B,* der(4)t(4;9)(p16.1;p23~p24) observed in patient 12. *C,* der(X)t(X;9)(p11;q31) from patient 13. Other unbalanced rearrangements observed included ins(8;5)(q24.1;q11.2q22) (patient 14 [*D*]), der(20)t(6;20)(p24;p13) (patient 15 [*E*]), and der(10)t(10;12)(q26;q24.1) (patient 16 [*F*]). For explanation of the classification colors at the p arms of both chromosomes 13 (*A*) and at the centromeres of chromosomes 9 (*B* and *C*) and 20 (*E*), as well as the additional color at translocation sites in *B, D,* and *E,* see the text.

of the idic(15) chromosomes (figs. 1*C* and2*A*). These regions consist of heterochromatin, and hybridization is suppressed by the addition of Cot-1 DNA to the M-FISH probe mix. However, weak FISH signals in these regions can result in the generation of classification colors that, in fact, are merely artifacts.

Both the history of patient 3, who had cat-eye syndrome, and the results of the Ag-NOR staining, which showed two signals at either end of a de novo marker chromosome, were suggestive of the presence of the typical bisatellited isodicentric chromosome with copies of the 22pter→22q11.2 region (McDermid et al. 1986). As expected, the SMC displayed the chromosome 22–specific spectral signature, and, in good concordance with the results from Ag-NOR staining, no hybridization signals were observed at either end of the marker chromosome (2*B*). The commercial LSI DiGeorge/VCFS probe (22q11.2) did not hybridize to the marker chromosome (data not shown), thereby refining the karyotype to 47,XX,+psu idic(22)(q11.21).

In patients 4 and 5, who had features of Turner syndrome, a mosaicism (45,X/46,X,+mar) was found by banding analysis. In each case, the marker chromosomes were de novo, DA/DAPI-negative, isodicentric, and contained Y-chromosome material in the M-FISH analysis (2*C*and *D*). When these results were considered together with those from CBG-banding analysis, the karyotypes were defined as 45,X/46,X,psu idic(Y)(q11.2).

Patient 6 was referred for prenatal diagnosis because of advanced maternal age and a history of three miscarriages. All metaphase spreads in the amniotic-fluid culture showed a de novo SMC. The M-FISH analysis unveiled chromosome 18 euchromatin in this SMC (2*E*). Involvement of only 18p was shown, by 18p- and 18q-specific DNA probes. The signal on the marker chromosome was smaller than the signals on both homologue chromosomes 18, suggesting a partial trisomy 18p. The marker chromosome was also evaluated by reverse painting. This allowed the exact mapping of the 18p material, to bands 18pter–18p11.2, and revealed that the centromere of the SMC was derived from chromosome 21. Thus, the karyotype was defined as 47,XX,+der(21)t(18;21)(p11.2;q11.1).

In patients 7–9, de novo SMCs showed no M-FISH signal (fig. 3). Two samples from prenatal diagnosis were prepared from direct preparations of chorionic villi (patients 7 and 8). Patient 9 was an adult male whose chromosomes were checked as part of a series of investigations for infertility. The absence of any fluorescence signal suggested that these SMCs consisted solely of heterochromatin, since any hybridization to these regions would be suppressed by the addition of unlabeled Cot-1 DNA to the hybridization mix. This was confirmed in patients 7 and 8, by reverse painting. In patient 7, only the centromere of chromosome 7 showed a signal that

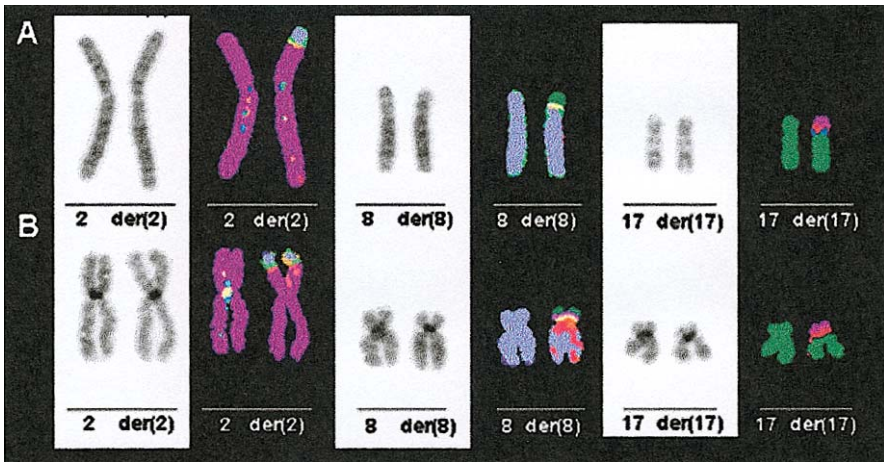


Figure 6 Identification of the same balanced complex translocation t(2;17;8)(p23;p11.2;p12) observed in a mother (patient 17 [*A*]) and her fetus (patient 18 [*B*]). The initial GTG-banding had identified a t(2p;8p), and the additional involvement of chromosome 17 was unraveled by M-FISH. In a display similar to that of figure 5, the inverted DAPI images are shown in the first, third, and fifth columns, and the respective chromosomes are shown, in classification colors, in the second, fourth, and sixth columns. The nonhomogeneous classification of chromosome 8 material in the der(8) (*B*) was caused by an adjacent chromosome. The occurrence of the additional color generated at the site of translocation breakpoints is explained in the text.

refined the karyotype to 47,XY,+der(7)(:p11→q11.1:). In patient 8, the short arms of all acrocentric chromosomes were stained, making additional hybridizations with chromosome-specific probes necessary. These hybridizations revealed that the marker was derived from either chromosome 14 or chromosome 22, resulting in the karyotype 47,XY,+i(14 or 22)(p10) (data not shown).

Interchromosomal rearrangements.—In patient 10, a female with a history of recurrent abortions, a balanced translocation, t(1;21)(p36.3;q22.1) was noted. Figure 4 illustrates the ability of M-FISH to detect such a small translocation and demonstrates the effect that chromosome condensation has for the detection of subtle alterations. Although the translocation can easily be identified on long chromosomes (fig. 4*A*), detection becomes more difficult with increasing condensation (fig. 4*B*). When the hybridization patterns on the translocation chromosomes were compared with the DAPI images, the breakpoint on the der(21) was refined, to subband 21q22.3. To verify whether the fetus had a balanced or unbalanced karyotype, FISH was performed on metaphase spreads from placental villi, and it confirmed the presence of the same constitutional anomaly.

De novo structural abnormalities on 4p were noted in patients 11 and 12. Patient 11 was an infant with clinical features of Wolf-Hirschhorn syndrome. GTG-banding revealed additional material of unknown origin, which replaced segment 4p15.2-pter. M-FISH discerned chromosome 13 material at the distal tip of the short arm of der(4) (fig. 5*A*), thereby redefining the karyotype to 46,XX,der(4)t(4;13)(p15;q32). In patient 12, a del(4)(p16) prompted use of an M-FISH analysis, to assess whether the loss of chromosome 4p material was the result of an unbalanced translocation. M-FISH revealed an additional hybridization band in the relevant 4p region (fig. 5*B*). However, the spectral signature was ambiguous, indicating the involvement of either chromosome X material or chromosome 9 material. This result guided the probe selection for additional experiments, deciphering the additional material as being derived from 9p. Thus, the karyotype was refined to 46,XX,der(4)t(4;9)(p16.1;p23~p24). As mentioned above with regard to patients 1 and 2, the p arms of acrocentric chromosomes cannot be evaluated, because of the suppression conditions. The same is true for large heterochromatic blocks, such as the centromeres of chromosomes 9. Thus, the classification colors at the p arms of both chromosomes 13 (fig. 5*A*) and at the centromeres of chromosome 9 (fig. 5*B*) are artifacts caused by very weak fluorescence signals. An additional color can be generated at the site of translocation breakpoints (fig. 5*B*). This is caused by the already described blending of colors, which is due to fluorescence flaring at the junctions of the individual chromosome painting–probe domains (Speicher et al. 1996).

Patient 13 was a female who presented with both aplasia of the thymus and cardiac malformations. GTG-banding showed that de novo material of unknown origin had replaced chromosome bands Xp11-pter. M-FISH revealed that this material was from chromosome 9 (fig. 5*C*), and subsequent hybridizations with chro-

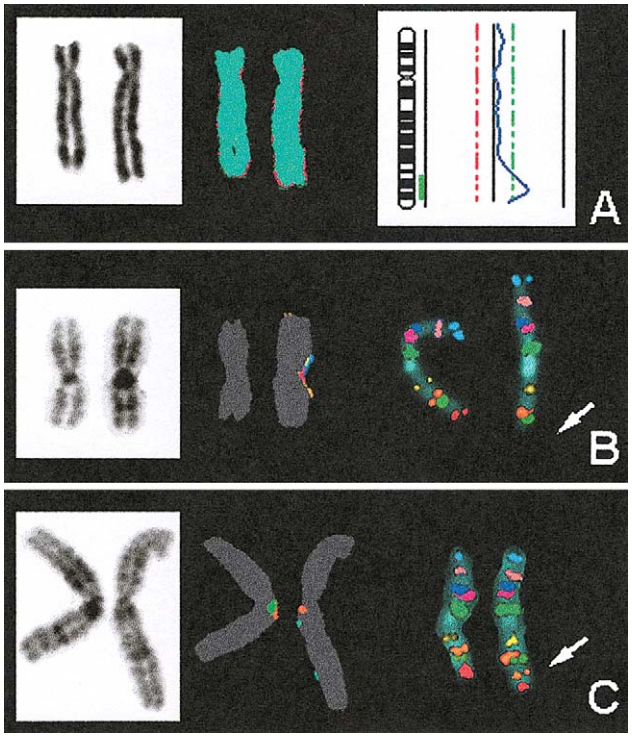


Figure 7 M-FISH and additional FISH experiments to decipher intrachromosomal rearrangements. *A*, De novo elongation of the long arm of a chromosome 4 (patient 19 [*first column*]). M-FISH painted the entire der(4) chromosome homogeneously, indicating a duplication of chromosome 4 material (*second column*). The duplicated material was mapped, by CGH, to chromosome bands 4q31-q35 (*third column*). *B* and *C*, G-banding in patients 20 and 21, which revealed structural abnormalities on the long arm of chromosome 1, resulting in small size differences: a shortening in patient 20 (*B, first column*) and an elongation in patient 21 (*C, first column*). In M-FISH, the chromosomes 1 displayed the spectral signature for chromosome 1 only (*B, second column;* and *C, second column*). Application of a multicolor chromosome 1–specific bar code in a second hybridization allowed the correct identification and mapping of the deletion and duplication, respectively (*B, third column;* and *C, third column*). In patient 20 (*B*), the 1q43-44 band–specific YAC (*red*) was missing on the der(1) (*arrow*), whereas all other YACs displayed the expected signals. Thus, the der(1) was defined as del(1)(q42). In patient 21 (*C*), a second signal for the YAC specific for band 1q32 (*brown*) was visible (*arrow*) between YACs specific for bands 1q41 (*green*) and 1q43-q44 (*red*), allowing us to define the der(1) as ins(1)(q42q41q32.1).

mosome arm–specific probes allowed us to refine the karyotype to 46,X,der(X)t(X;9)(p11;q31). The red classification color occurs at the location of the centromeres of both chromosomes 9 and, as in figure 5*B*, is caused by weak hybridization signals.

GTG-banding diagnosed karyotype 46,XY,add(8)(q24) in patient 14, who had dysmorphic features and cardiac anomalies. M-FISH indicated insertion of chromosome 5 material into band 8q24 (fig. 5*D*). Fluorescence flaring caused a blending of colors, resulting in an additional band at the site of the insertion (see explanation above; also see Speicher et al. 1996). For verification, an 8q subtelomeric cosmid (489D14) was hybridized in a second experiment, and it resulted in signals on the long arms of both the der(8) and the normal chromosome 8. The karyotype was refined to 46,XY,ins(8;5)(q24.1;q11.2q22).

Karyotype analysis in patient 15, who had dysmorphic features, showed de novo karyotype 46,XX,add(20)(p13). M-FISH delineated this additional material as being derived from chromosome 6 (fig. 5*E*). To map the involved region of chromosome 6 and to check whether chromosome 20 material was lost, additional experiments with microdissected probes 6pter-6p24 and 6q25.3-6qter and the subtelomere cosmid 2005a4, which is specific for 20p, were performed. These experiments showed that 6p material contributed to the der(20) and that the 20p subtelomeric region was lost. Thus, the karyotype was altered to 46,XX,der(20)t(6;20)(p24;p13).

In patient 16, the karyotype of cultured amniotic-fluid cells was 46,XX,add(10)(q26), and M-FISH identified the additional material as being a segment from chromosome 12 (fig. 5*F*). Reverse painting corroborated the M-FISH result and, in addition, allowed a precise mapping of the chromosome 12 material, resulting in the karyotype 46,XX,der(10)t(10;12)(q26;q24.1).

In patients 17 and 18 (amniotic-fluid culture from the child [patient 18] and peripheral blood from the mother [patient 17]), a balanced translocation t(2p;8p) was perceived by GTG-banding. The banding pattern on the p arm of the aberrant chromosome 8 was atypical and did not correspond to the expected distal end of the short arm of chromosome 2. M-FISH was initially requested on metaphase spreads of the mother only, to rule out the involvement of a third chromosome and for a precise breakpoint mapping on chromosomes 2 and 8 (patient

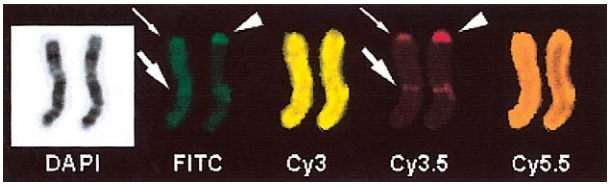
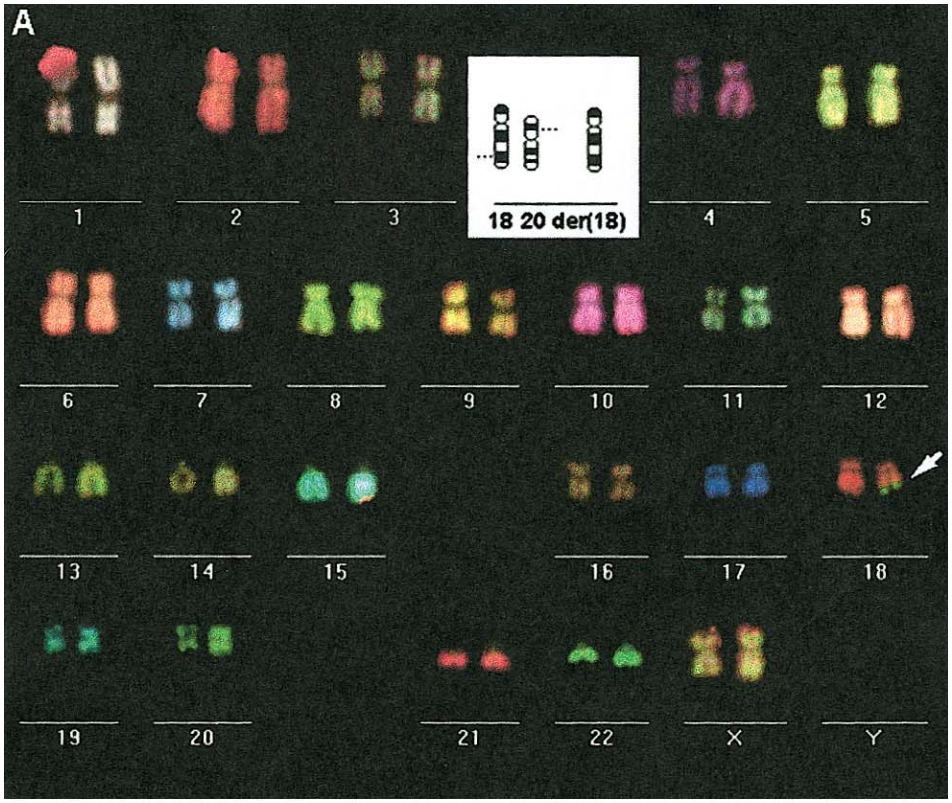


Figure 8 Hybridization pattern on the two X chromosomes in a 46,XX male (patient 23). The different labeling of the X chromosome (Cy3 and Cy5.5) versus the Y chromosome (FITC, Cy3.5, and Cy5.5) in the M-FISH mix results in additional bands on the X chromosome. These bands are visible in the FITC and Cy3.5 channels and represent the first pseudoautosomal region at Xp22.3 (size 2.6 Mb [*smaller arrows*]) and the XY homology region at Xq21.3 (size 4 Mb [*larger arrows*]). The second pseudoautosomal region at Xq28 is too small (320 kb) to be detected. Translocation of Y-chromosome euchromatin to one of the X chromosomes resulted in a wider band at the first pseudoautosomal region (*arrowhead*).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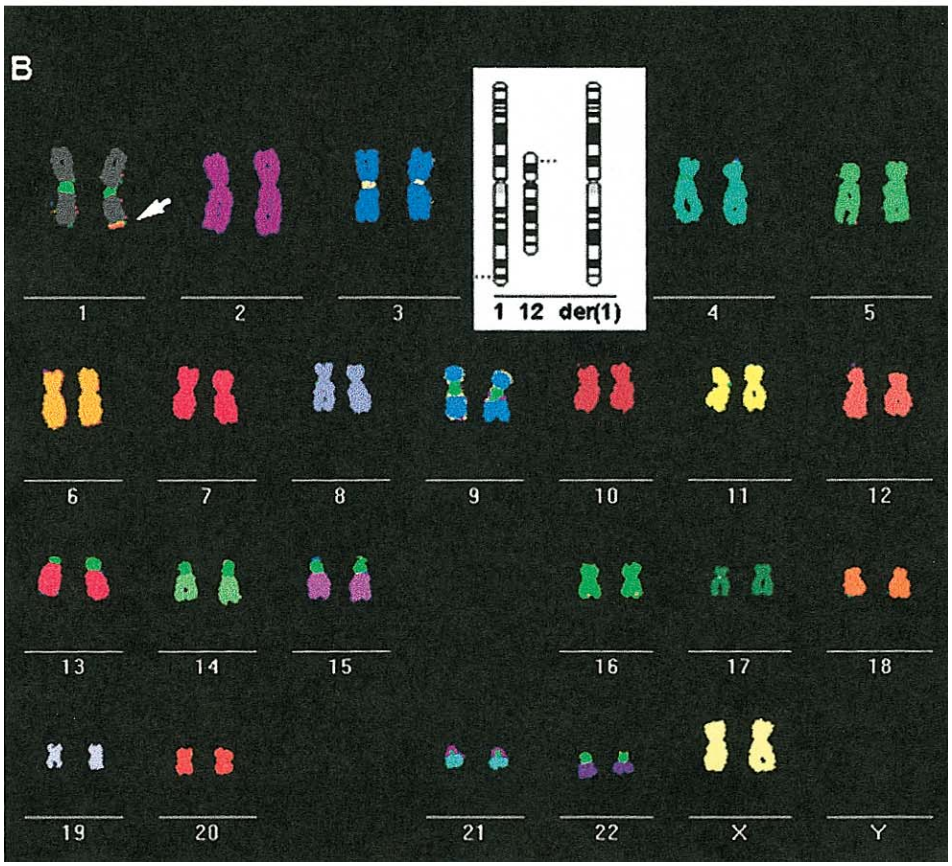
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17). Indeed, M-FISH could identify chromosome 17 as being involved in a complex translocation (fig. 6*A*). This prompted an analysis of the chromosomes of the fetus (patient 18; see fig. 6*B*). Within the resolution limits of M-FISH, the complex translocation in the fetus appeared to be the same as that in the mother. Fine mapping of breakpoints on prometaphase chromosomes from the mother yielded karyotype 46,XX,t(2;17;8)(p23;p11.2;p12). The additional color generated at the site of translocation breakpoints was due to blending of colors, caused by fluorescence flaring, as already has been mentioned for patients 11 and 12.

Intrachromosomal rearrangements.—In patient 19, banding analysis demonstrated a de novo add(4)(q33). M-FISH showed the der(4) to be homogeneously painted with chromosome 4 material, ruling out an interchromosomal translocation or insertion (fig. 7*A*). The duplicated region was mapped by CGH, resulting in refinement of the karyotype to 46,XX,dup(4)(q31q35).

Both patient 20 and patient 21 presented with dysmorphic features and cardiac defects. In each case, banding analysis revealed a der(1) caused by shortening (patient 20) or elongation (patient 21) of the long arm (fig. 7*B* and *C*). After M-FISH, both der(1) chromosomes displayed the chromosome 1 spectral signature along their entire length, without any sign of a translocation. In fact, the size differences between the two chromosomes 1 were, in both cases, so minor that, without the information from banding analysis, the M-FISH results alone could have been interpreted as "normal."

A multicolor chromosome 1–specific bar code was applied, to analyze the intrachromosomal rearrangements in more detail. In both cases, YACs specific for bands 1p36.3-1q41 showed the expected hybridization patterns (fig. 7*B* and *C*). In patient 20, the 1q43-q44–specific YAC (yRM2123) displayed no signal on the der(1) (fig. 7*B*). Thus, this karyotype was refined to 46,XX,del(1)(q42). In patient 21, the 1q43-q44–specific YAC (yRM2123) hybridized to the q-terminal end of the der(1), resulting in a greater distance to the 1q41-specific YAC (958e1), compared with that in the normal chromosome 1 (fig. 7*C*). Between these two YACs, a signal for the 1q32-band–specific YAC (773c6) was visible. Thus, chromosome band 1q32 was duplicated and inserted between 1q42 and 1q43 (fig. 7*C*). On the basis of this information, the GTG-banding pattern was reevaluated, and the der(1) was defined as ins(1)(q42q41q32.1).

In patient 22, who was one child of a twin pregnancy, prenatal diagnosis was requested because abnormal growth was noted by ultrasound. G-banding of metaphase spreads from amniotic-fluid cells of this child revealed a de novo add(13)(p11). M-FISH deciphered this material as being from chromosome 13 (data not shown), yielding the karyotype 46,XY,der(13)(qter→q21::p11→qter).

XX males.—In patients 23 and 24, two 46,XX males, M-FISH analysis was performed to test whether parts of the Y chromosome could be detected. Both patients had some clinical features of Klinefelter syndrome, and banding analysis showed a female karyotype (46,XX). M-FISH detected Y-chromosome material in the telomeric region of the p arm of one X chromosome in patient 23 (fig. 8), refining the karyotype to 46,X, der(X)t(X;Y)(22.3;p11.3). In patient 24, the M-FISH result was consistent with a normal female karyotype, and no Y-chromosome material was detectable.

Normal Karyotype by GTG-Banding but Phenotype Suggestive of Chromosomal Syndrome

Twenty patients with mental retardation and dysmorphic features with a normal GTG-band– karyotype were screened. In two of these patients, an unbalanced translocation was revealed. In patient 25, M-FISH identified a der(18) resulting from an unbalanced t(18;20) (fig. 9*A*). Experiments with region-specific probes allowed both mapping of the breakpoints and revision of the karyotype to 46,XX,der(18)t(18;20)(q21;p11.2). Subsequently, the chromosomes of the parents were analyzed. The chromosomes of the mother were found to correspond to a normal female karyotype; a balanced t(18;20)(q21;p11.2) was found in the father (data not shown). In patient 26, M-FISH could identify an unbalanced translocation t(1;12) (fig. 9*B*). Subsequent hybridizations with subtelomeric probes specific for 1q, 12p, and 12q further refined the karyotype, to 46,XX,der(1)t(1;12)(q43;p13). The mother was found

Figure 9 Cases in which M-FISH could identify unbalanced translocations in patients with dysmorphic features and mental retardation and with a karyotype classified as normal on the basis of GTG-banding. *A,* patient 25, in whom M-FISH identified a der(18)t(18;20)(q21;p11.2) (*arrow*). The karyogram is displayed in true colors, to facilitate visualization of the translocation, because chromosomes 18 and 20 have similar classification colors. The spot visible on the short arm of the left chromosome 1 represents unspecific background on the slide. A diagrammatic representation of the involved chromosomes is shown in the inset: the normal chromosomes 18 and 20, with breakpoints indicated by dotted lines, are on the left; the der(18), for which the t(18;20) results in a banding pattern identical to that of the normal 18, is on the right. *B,* der(1)t(1;12)(q43;p13) identified in patient 26 (*arrow*). As shown in the inset, the exchange of chromosome 1 material and chromosome 12 material resulted in a minor alteration of the banding pattern in the der(1). For an explanation of the classification colors observed at some centromeric regions and at the short arms of the acrocentric chromosomes, see the text.

to be a carrier of a balanced translocation, t(1;12). It is important to note that, in both cases, the chromosomes of the parents and child were screened by at least two outside laboratories and that, in each examination, no abnormalities were found. The interpretation of the classification colors observed at some centromeric regions and at the short arms of the acrocentric chromosomes infig. *9B* have been explained above.

Discussion

M-FISH has proved to be a very reliable procedure that has now been applied to many slides ($n = 121$) contributed by a number of different laboratories ($n = 18$). In all cases, results were obtained without any special slide-preparation techniques being required. In fact, M-FISH was applied to metaphase spreads of various ages, from freshly prepared (<24 h) to several weeks old. Variations in the slide pretreatment, depending on the amount of cytoplasm present, and in the denaturation time (see the Material and Methods section) are important for success. M-FISH works equally well on samples derived from direct preparations of chorionic villi, amniotic-fluid cells, lymphocytes, or fibroblasts. In the following, we will discuss both the application of M-FISH in different diagnostic settings and the interpretation of results. A hierarchical approach to chromosome analysis is presented. The first step in this approach is the application of M-FISH, and subsequent steps can include CGH, chromosome-bar coding, locus-specific hybridization, and reverse painting. The initial M-FISH experiment determines the appropriate subsequent step(s) to take. As shown in the last three columns of table 1, this procedure provides more-accurate identification of cytogenetic abnormalities than can be obtained by GTG-banding.

Cytogenetic Abnormalities

SMCs.—Most marker chromosomes found in pre- and postnatal diagnostic situations are SMCs, small structurally abnormal chromosomes that occur in addition to the 46 normal chromosomes. The incidence of SMCs in the general population is 1/3,000 (Buckton et al. 1985), with the vast majority being derived from acrocentric chromosomes, mostly chromosome 15 (Friedrich and Nielsen 1974; Buckton et al. 1985). To determine whether an SMC contains euchromatin with possible detrimental effects for the patient, many investigators have used FISH (e.g., see Callen et al. 1990; Rauch et al. 1992; Blennow et al. 1993; Plattner et al. 1993; Crolla et al. 1997, 1998). Here, we have demonstrated the role of M-FISH in both the detection of small euchromatic regions and the deciphering of their chromosomal origins. Depending on the results, additional FISH experiments with region-specific probes may be necessary to define conceivable adverse phenotypic effects more precisely. For example, in patient 6 a second hybridization was performed to determine the involvement of the p or q arm of chromosome 18.

In light of the fact that most SMCs are derived from the short arms of acrocentric chromosomes, it is not surprising that no specific hybridization signal was observed in some patients (patients 7–9; fig. 3). Hybridization to SMCs composed of only heterochromatin would be expected to be completely suppressed, because of the addition of Cot-1 DNA to the hybridization mix. This was confirmed by simultaneous microdissection experiments. Thus, M-FISH can assist in the determination of whether an SMC consists solely of heterochromatin. We have decided to describe these SMCs as der(?)(h), to indicate that the origin of the centromere is unknown but that the marker chromosome consists only of heterochromatin. However, a diagnosis based on a "missing" signal is problematic in cases of a poor hybridization efficiency or a poorly prepared probe set. In addition, the lack of M-FISH signals does not contribute in the determination of the origin of the SMC. Here, reverse painting has the advantage of mapping the origin of SMCs accurately. However, it is important to note that neither technique can guarantee the detection of very small euchromatic regions. An attractive addition to the analysis of SMCs would be the development of a multicolor centromere-probe set capable of staining all centromeres in different colors.

Inter- and intrachromosomal rearrangements.—M-FISH is especially powerful in cases in which structurally abnormal chromosomes are observed. M-FISH can easily distinguish between inter- and intrachromosomal rearrangements. In cases of balanced translocations, M-FISH allows the breakpoints to be mapped with great accuracy. In contrast, unbalanced translocations often require additional FISH experiments, in order to narrow the chromosomal segment participating in the structural rearrangement. For example, in patient 15, M-FISH deciphered the additional material on chromosome 20 as being derived from chromosome 6 (fig. *5E*). The translocated material was small and lacked a characteristic banding pattern, making a second hybridization with chromosome 6 region–specific probes necessary, in order to pinpoint which region resulted in the partial trisomy 6. In addition, neither DAPI- nor GTG-bands allowed a determination on whether material from the short arm of chromosome 20 was missing.

In cases of small intrachromosomal rearrangements, the resolution limits of GTG-banding are superior. In patients 20 and 21, for example, structural rearrangements on the long arm of chromosome 1 were noted by banding analysis. M-FISH excluded a translocation, but, in both cases, the structural rearrangements resulted in

such minor size differences between the two homologous chromosomes that the karyotypes based on M-FISH alone would have been misclassified as normal (fig. 7*B* and *C*). Thus, classical G-banding remains indispensable as a first screening test for the integrity of the genome.

Occasionally, if the translocated material is small, the classification of the spectral signature might be ambiguous (as in patient 12; see fig. 5*B*). This problem is not system dependent and has also been reported in the spectral karyotyping system (Schröck et al. 1997).

The aforementioned examples demonstrate that subsequent FISH experiments are often required, in order to characterize the observed changes in greater detail. In these cases, M-FISH serves as a guide to the selection of the optimal procedure, such as reverse painting, CGH, or application of defined region-specific probes. The advantages of reverse painting include the mapping of euchromatin in one experiment (patients 6 and 16). On the other hand, this procedure depends on the experience and skill of the operator. CGH allowed the mapping of the overrepresented region on the long arm of chromosome 4 in patient 19 (fig. 7*A*). However, CGH is time-consuming, because of the requirement of DNA preparation, the longer hybridization times, and the requirement of quantitative analysis of results. Multicolor chromosome-specific bar codes have several advantages (Lengauer et al. 1993). They allow the accurate mapping of over- or underrepresented regions (fig. 7*B* and *C*). In addition, they yield information on mosaicism, which may be difficult to gain by other methods, such as CGH (since it relies on DNA from the total cell population) or microdissection. Moreover, bar codes unravel structural rearrangements, such as inversions, that cannot be obtained by other methods (patient 21 [fig. 7*C*]; S. Schuffenhauer, S. Uhrig, and M. R. Speicher, unpublished observations). Bar codes can easily be constructed from cytogenetically and genetically anchored CEPH YACs, which are publicly available (e.g., from Fondation Jean Dausset CEPH or the Max Planck Institute of Molecular Genetics. The resolution of the bar codes can be tailored for specific questions, on the basis of the number of YAC clones used simultaneously.

XX males.—We tested M-FISH in two individuals who had a male phenotype but who, on the basis of GTG-banding analysis, had a normal 46,XX karyotype. Because the sequence essential for sex determination may be as small as 35 kb, alterations may, in many cases, be below the M-FISH resolution limit. In one patient, chromosome-Y material was detected at the distal end of the short arm of one X chromosome (patient 23; see fig. 8). This went undetected by G-banding analysis, because it did not change the banding pattern of the X chromosome.

Normal Karyotype by GTG-Banding but Phenotype Suggestive of Chromosomal Syndrome

We started to use M-FISH to screen individuals with mental retardation and dysmorphic features but without any karyotypic abnormality detected by G-banding. We were able to detect unbalanced translocations in 2 (patients 25 and 26) of 20 patients (fig. 9). Thus, the screening of defined groups of patients by M-FISH may be rewarding. Although M-FISH alone seems to be powerful for the detection of unbalanced translocations, a "normal" result should not be interpreted as definite exclusion of a translocation. The exact resolution limits are difficult to determine. In general, the X chromosome yields an approximate measure for both the resolution and the hybridization quality. The consistent detection of the first pseudoautosomal region at Xp22.3, in conjunction with the concurrent absence of the second at Xq28 (fig. 8), sets the resolution limits at 320 kb–2.6 Mb. However, as shown in figure 4, the resolution limit is also influenced by both chromosome condensation and the fluor composition of chromosomes participating in structural abnormalities. Thus, the resolution cannot be given in absolute numbers. In general, an improvement of resolution can be achieved only by switching from painting probes to sets of well defined region-specific probes. For example, subtelomere probes (Ning et al. 1996) should be more sensitive for the detection of subtle translocations (Ledbetter 1992) but would not detect intrachromosomal rearrangements. The potential that subtelomere probes and the bar-code approach have for the improvement of resolution emphasize that the use of a multicolor system should not be restricted to the application of whole chromosome–painting probes alone.

In this article we have demonstrated the use of M-FISH in pre- and postnatal diagnostic applications, on a routine basis. The strength of M-FISH lies in the characterization of marker chromosomes and other structural abnormalities. The finding of cryptic unbalanced translocations in patients with dysmorphic features suggests that the screening of "normal" metaphase spreads of defined groups of patients by means of M-FISH may be a rewarding addition to G-banding analysis. Recent improvements of the M-FISH procedure (Eils et al. 1998; Bolzer et al. 1999) allow results in <24 h, which is especially important for prenatal diagnostic applications.

After M-FISH, further refinements of a karyotype are often needed, requiring additional FISH applications with region-specific probes. To do this, well-defined probe sets, such as subtelomere probes and chromosome-specific multicolor bar codes, which we have established for a number of chromosomes, are the ideal tools. Application of these tools results in a hierarchical

approach of chromosome analysis, yielding a stepwise improvement in the accurate designation of karyotypes, within the maximum achievable cytogenetic resolution. This is a prerequisite to the establishment of the best possible correlation between phenotype and genotype and for the improvement of genetic counseling.

Acknowledgments

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Electronic-Database Information

URLs for data in this article are as follows:

CEPH, http://www.cephb.fr/bio (for YAC clones)
Max Planck Institute of Molecular Genetics, http://www.mpimg-berlin-dahlem.mpg.de/ (for YAC clones)

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