Am. J. Hum. Genet. 66:1684–1688, 2000

Report

An Optimized Probe Set for the Detection of Small Interchromosomal Aberrations by Use of 24-Color FISH

Jorge Azofeifa,[1,3] Christine Fauth,[1] Jürgen Kraus,[1] Christine Maierhofer,[1] Sabine Langer,[1] Andreas Bolzer,[1] Jay Reichman,[4] Simone Schuffenhauer,[2] and Michael R. Speicher[1]

[1]Institut für Anthropologie und Humangenetik and [2]Abteilung für Medizinische Genetik der Kinderklinik, Ludwig-Maximilians-Universität München, Munich; [3]Instituto de Investigaciones en Salud and Escuela de Biología, Universidad de Costa Rica, San José; and [4]Chroma Technology Corporation, Brattleboro, VT

The rapid spread of the use of new 24-color karyotyping techniques has preceded their standardization. This is best documented by the fact that the exact resolution limits have not yet been defined. Indeed, it is shown here that a substantial proportion of interchromosomal aberrations will be missed by all multicolor karyotyping systems currently in use. We demonstrate that both the sensitivity and the specificity of 24-color karyotyping critically depend on the fluorochrome composition of chromosomes involved in an interchromosomal rearrangement. As a solution, we introduce a conceptual change in probe labeling. Seven-fluorochrome sets that overcome many of the current limitations are described, and examples of their applications are shown. The criteria presented here for an optimized probe-set design and for the estimation of resolution limits should have important consequences for pre- and postnatal diagnostics and for research applications.

One of the most important applications of 24-color karyotyping, known as "multiplex-FISH" (M-FISH; Speicher et al. 1996) or "spectral karyotyping" (SKY; Schröck et al. 1996), is the detection and correct classification of small interchromosomal aberrations, which cannot be deciphered by means of GTG banding alone. The detection of these rearrangements should be highly sensitive, and their chromosomal origin should be recognized with high specificity. However, as shown in figure 1, the currently used multicolor systems do not fulfill these criteria. Interchromosomal aberrations result in color combinations that can be divided into three different types, on the basis of the sensitivity and specificity of their detection in 24-color karyotyping. For simplicity, in figure 1, these types of color combinations are shown for translocations only; however, the same considerations also apply to other structural aberrations, such as insertions. Translocations with type 1 and type 2 color combinations are detected with high sensitivity. Small translocations with a type 3 color combination are difficult or impossible to detect (fig. 1*b*) and thus pose a tremendous challenge for diagnostic applications.

The number of translocations that may result in a type 3 color combination is substantial. In a human male metaphase spread with 46 chromosomes, there are 552 possible two-way translocations. With use of a combinatorial five-fluorochrome labeling scheme (as done by Schröck et al. [1996] and Speicher et al. [1996]), there are $\geqslant$74 (13.5%) possible type 3 color combinations. Thus, the traditionally used multicolor mixes made of five fluorochromes are likely to miss a large number of small aberrations.

In the present study, we present a conceptual change in probe labeling, to reduce type 3 color combinations to a minimum. To avoid type 3 color combinations completely, each chromosome has to be labeled with the same number of fluorochromes. Since 24 different spectrally resolvable fluorochromes are not available, it is impossible to tag each chromosome with only one fluorochrome. With eight fluorochromes, there are 28 different double combinations, which allows for two-fluorochrome labeling of each chromosome; however, because the latest generation of fluorescence microscopes is equipped with eight-filter wheels, with one

Received January 11, 2000; accepted for publication February 28, 2000; electronically published April 4, 2000.

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

0002-9297/2000/6605-0023$02.00

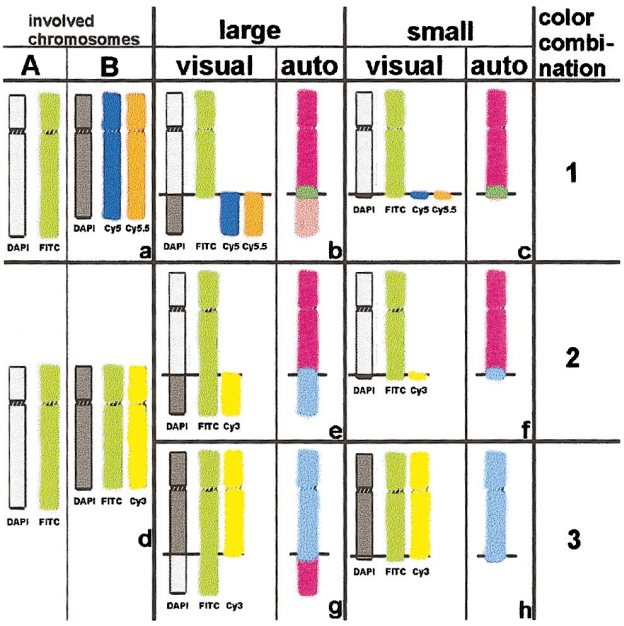

Figure 1 Sensitivity and specificity in 24-color karyotyping critically depend on the color combination on derivative chromosomes. Three different color-combination types are shown schematically, for large and small translocations. Only one translocation chromosome is discussed. *a–c,* In the type 1 color combination, both the translocated segment and the centric segment are labeled with at least one different fluorochrome. Chromosome A is labeled with FITC only; chromosome B is labeled with a combination of Cy5 and Cy5.5. The translocation can easily be identified by visual inspection (*b* and *c, visual*) of the individual channels. In the automated-classification image (*b* and *c, auto*), an additional color at the site of translocation breakpoints can be caused by the blending of colors through fluorescence flaring. This band's size depends mainly on chromosome condensation. If the translocated material is very small (*c*), then only the additional color may be visible on the image (*auto*). Type 1 color combinations always result in a color change and, therefore, will be detected with high sensitivity. Very small translocations (*c*) have a low specificity (*auto*) and may be misclassified. *d,* Chromosome A is labeled with FITC only, and chromosome B is labeled with a combination of FITC and Cy3, to illustrate the *second* and *third* color combinations. *e* and *f,* Translocation of chromosome B material to chromosome A results in a type 2 color combination. The centric segment is labeled with a subset of fluorochromes that occur in the translocated segment. No blending of colors is observed at translocation breakpoints, and there is usually a sharp transition between the two fused segments (*e* and *f, auto*). Because of fluorescence flaring, however, the breakpoint may erroneously appear to be too proximal. The extent of this breakpoint displacement depends mainly on chromosome condensation. Even a small translocation is usually correctly classified, because no color blurring occurs (*f*). In small translocations, it can sometimes be problematic to distinguish between a type 1 (*c*) or type 2 (*f*) color combination. *g* and *h,* Translocation of chromosome A material to chromosome B results in a type 3 color combination. The translocated segment is labeled with a subset of fluorochromes that occur in the centric segment—that is, it has no fluorochrome of its own. Because of fluorescence flaring, a breakpoint may falsely appear to be too distal (*g* and *h, auto*). This is fatal in very small translocations (*h*), since the flaring may completely obscure the translocation. Thus, panel *h* depicts the most challenging situation in 24-color karyotyping, since both sensitivity and specificity are low.

position needed for the visualization of the 4,6-diamidino-2-phenylindole (DAPI) counterstain, only seven fluorochromes can currently be used, on a routine basis, for probe labeling. With seven fluorochromes, each chromosome can be labeled with three fluorochromes, since there are 35 different triple combinations. However, such a probe set would significantly increase probe complexity. Therefore, additional seven-fluorochrome sets were designed. The first probe set had a minimal complexity, since seven painting probes were labeled with only one fluorochrome and since 17 painting probes were labeled with two fluorochromes (probe set a; fig. 2*a*). This probe mix reduces to 34 (6.2%) the number of possible type 3 color combinations in a male metaphase spread. There are two other options for seven-fluorochrome probeset designs. For both options, 21 chromosomes are labeled with all possible double-fluorochrome combinations, but the options differ with regard to the labeling of the remaining three chromosomes. Labeling each of these remaining three chromosomes with one fluorochrome (probe set b; fig. 2*b*) reduces the number of type 3 color combinations to 18 (3.3%) and 12 (2.4%) in male and female metaphase spreads, respectively. If necessary, only three painting probes in a male metaphase spread (or two painting probes in a female metaphase spread) are needed to exclude the occurrence of one of the remaining 18 and 12 type 3 color combinations in male and female metaphase spreads, respectively, in a second experiment.

Labeling each of the three remaining chromosomes with a triple combination (probe set c, not shown) further reduces the number of type 3 color combinations to nine (1.6%) and six (1.2%) in male and female metaphase spreads, respectively; however, the triple labels increase to seven the number of painting probes needed, in subsequent experiments, for the exclusion of hidden

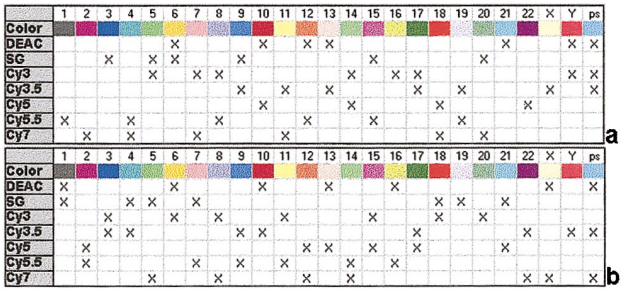

Figure 2 Labeling scheme with the color combinations for seven-fluorochrome probe sets a and b. *a* and *b,* Row 1 shows the chromosomes 1–22 and the two sex chromosomes X and Y. The ps column was added for PAR1 and XY-HR (compare with fig. 3*a*). The Color row depicts the pseudocolor look-up table. Rows 3–9 show the color combinations used in the experiments.

aberrations resulting from the type-3 color combinations. Therefore, only probe sets a and b were generated and were tested (1) on various metaphase spreads from pre- and postnatal patients referred to our clinical laboratory and (2) on the lung-cancer cell line A427 (purchased from the American Type Culture Collection).

Initially, the fluorochromes diethylaminocoumarine (DEAC/NEN) and SpectrumGreen (SG/Vysis) and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5, and Cy7 (Amersham Pharmacia Biotech) were used for probe labeling. In later experiments, Cy3.5 was replaced by TexasRed (Molecular Probes) and Cy7 was replaced by LaserPro IR790 (Molecular Probes). Hybridization, image capturing, and processing were done as described elsewhere (Eils et al. 1998).

Figure 3*a* demonstrates that landmarks on the X chromosome can be used for the assessment of hybridization quality and resolution limits. Different labeling of the X and Y chromosomes results in additional bands representing both the first pseudoautosomal region (PAR1; 2.6 Mb) at chromosome Xp22.3 and the XY homology region (XY-HR; 4 Mb) at chromosome Xq21.3. The second pseudoautosomal region (320 kb) at chromosome Xq28 could never be detected.

After hybridization of probe set a to a metaphase spread from the lung-cancer cell line A427, type 1 and type 2 color combinations were observed on some derivative chromosomes (fig. 3*b–d*). On two chromosomes 12, a small segment of chromosome 18 was translocated to the long arm. The translocation was either almost (fig. 3*b*) or completely (fig. 3*c*) misclassified, because of fluorescence flaring and color blending typically observed in type 1 color combinations (compare with fig. 1*c*); however, the origin of the translocated material can be checked by means of visual inspection of signals in the individual channels. Since chromosome 18 is labeled with Cy5 and Cy7, since chromosome 12 is labeled with DEAC and Cy5.5 (fig. 2*a*), and since no triple combinations are used, the signals are unequivocal and cannot be confused through flaring signals from the centric segment. This case shows the different specificity of *automated* versus *visual* classification in small type 1 color combinations.

A translocation tagged with a type 2 color combination is shown in figure 3*d* (compare with fig. 1*e* and *f*). Because of the fluorochrome composition of the two chromosome segments, color blending cannot occur. Thus, both sensitivity and specificity are high.

In a patient with a mild mental handicap, results of banding analysis revealed a small structural abnormality at the terminal end of chromosome 6q. Figure 4*a* and *b* depicts two chromosomes 6 of the same metaphase spread after hybridization of the traditional five-fluorochrome mix. Neither the classification result nor the inspection of individual channels gave any hint of the presence of an interchromosomal aberration in any of the 10 evaluated metaphase spreads. The results of subsequent experiments revealed that one of the two chromosomes 6 was, in fact, a der(6)t(6;16)(q27;p13.3). Labeling of chromosome 6 with fluorescein isothiocyanate (FITC), Cy5, and Cy5.5 and labeling of chromosome 16 with FITC and Cy5 resulted in a type 3 color combination on the der(6) (see fig. 1*h*). When seven-fluorochrome mix a was applied to the der(6)t(6;16), the translocation was readily visible (fig. 4*c* and *d*), since the translocated segment of chromosome 16 added an additional fluorochrome to the der(6).

Figure 4*e–j* shows the analysis of a balanced translocation t(2;3)(q37;p25). In the traditional five-fluorochrome mix, the translocation of chromosome 2 mate-

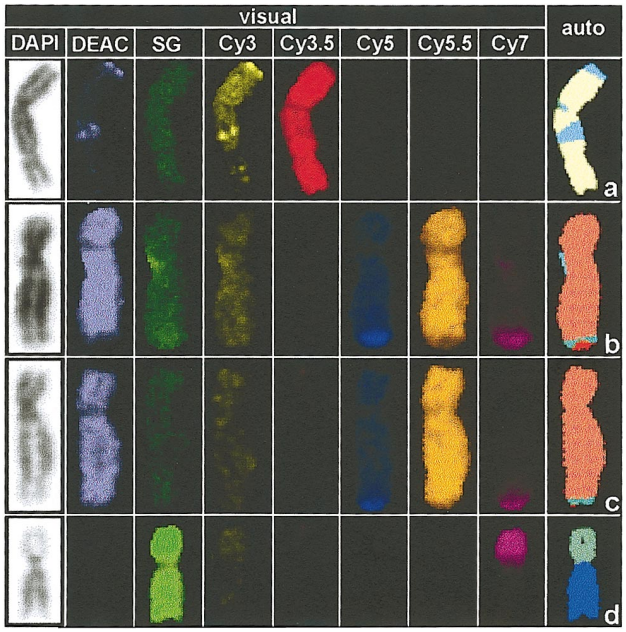

Figure 3 Exemplary classification results for some chromosomes generated after hybridization of seven-fluorochrome set a on lung-cancer cell line A427. All eight-fluorochrome channels are shown as they are used for visual classification (*visual*). The auto column depicts the result of the automated classification. *a*, Detail of the hybridization pattern of the X chromosome. Cross-hybridization of the Y chromosomes (labeled with DEAC and Cy3) to the X chromosomes (Cy3.5) is visible as bands representing the PAR1 at chromosome Xp22.3 (2.6 Mb) and the XY-HR at chromosome Xq21.3 (4 Mb). *b* and *c*, t(12;18)(q24.3;?), labeled with a type 1 color combination. Chromosome 12 is labeled with a combination of DEAC and Cy5.5, and chromosome 18 is labeled with a combination of Cy5 and Cy7. The small, translocated chromosome 18 segment is visible (*b*, *auto*) as a red band distal to a green band caused by color blending. It is, however, completely misclassified in panel *c*, because only color blending is visible (compare with fig. 1*c*). *d*, Type 2 color combination on a derivative chromosome consisting of chromosomes 20 (SpectrumGreen and Cy7) and 3 (SpectrumGreen only) material. Blending of colors at the junction of the two chromosomes cannot occur (compare with fig. 1*e*).

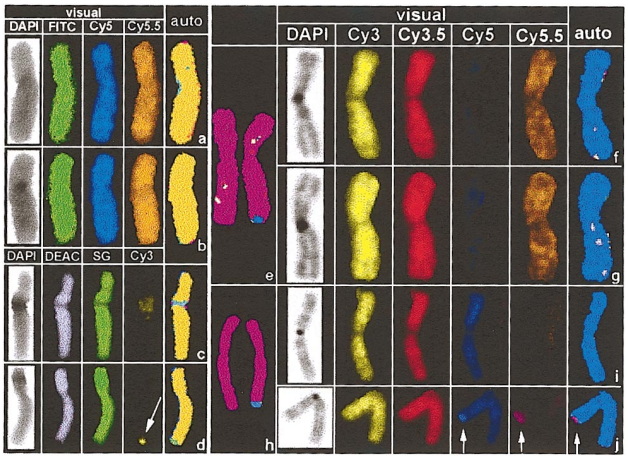

Figure 4 Two cases of small translocations resulting in type-3 color combinations. *a* and *b*, Analysis of an unbalanced translocation der(6)t(6;16)(q27;p13.3) with the traditional five-fluorochrome mix. The translocated chromosome 16 material (FITC and Cy5) does not add an additional fluorochrome to the derivative chromosome 6 (FITC, Cy5, and Cy5.5). The only difference between the normal and the derivative chromosome 6 should consist of a small size difference in the Cy5.5 channel. This is impossible to detect by visual inspection and is missed by the automated classification (compare with fig. 1*b*). Which of the two chromosomes 6 in *a* or *b* is normal and which is derivative cannot be concluded. *c* and *d*, Analysis of the der(6)t(6;16) with seven-fluorochrome set a. Chromosome 6 is labeled with fluorochromes DEAC and SpectrumGreen, and chromosome 16 is labeled with Cy3. The translocated material is readily visible in the Cy3 channel (*d*, *arrow*). The additional color band within the normal chromosome 6 (*c*) is caused by an overlapping chromosome. *e–g*, Analysis of a balanced translocation t(2;3)(q37;p25) with the traditional five-fluorochrome mix. Chromosome 2 is labeled with Cy5.5 only; chromosome 3 is labeled with a combination of fluorochromes Cy3, Cy3.5, and Cy5.5. The chromosome 3 material translocated to chromosome 2 is readily visible (*e*), but the chromosome 2 material translocated to chromosome 3 (*f* and *g*) is not. *h–j*, In seven-fluorochrome mix b, chromosome 2 was labeled with fluorochromes Cy5 and Cy5.5, and chromosome 3 was labeled with fluorochromes Cy3 and Cy3.5. This results in type 1 color combinations on both translocation chromosomes (arrows in *j* indicate translocated chromosome 2 material), allowing for the unequivocal classification of the rearrangement as balanced translocation.

rial to chromosome 3 results in a type 3 color combination, whereas the translocation of chromosome 3 material to chromosome 2 results in a type 1 color combination. As expected, the chromosome 3 material that was translocated to chromosome 2 was readily visible (fig. 4*e*), but the chromosome 2 material that was translocated to chromosome 3 (fig. 4*f* and *g*) was not. With the traditional five-fluorochrome mix, this balanced translocation would have been falsely interpreted as an unbalanced chromosomal rearrangement. In contrast, seven-fluorochrome mix b (fig. 2*b*) allowed for the unequivocal classification of this translocation (fig. 4*h–j*). In the two cases shown in figure 4, comparison with the PAR1 signal indicated that the size range of the translocated material was 3–5 Mb in each case.

Problems in the detection of small rearrangements are not confined to a filter-based system but, rather, can occur with use of the SKY system as well. This is best documented by the cross-hybridization pattern of the Y chromosome to the homologous PAR1 and XY-HR on the X chromosome, which usually is not reported in studies of SKY (e.g., Schröck et al. 1996, 1997; Macville et al. 1999). For discrepant detection of structural changes in clinical cases, a case in point is a report by Haddad et al. (1998), in which the PAR1 region was seen while, at the same time, the larger XY-HR was missed. The consistent failure to detect XY-HR disputes the claim of the SKY Consortium that the sensitivity of their system generally lies in a range of 1.5 Mb (Schröck et al. 1996; Haddad et al. 1998).

Even with the use of the probe sets described in the present study, some pitfalls remain. First, translocated material involving <2.6 Mb will be difficult to detect (Holinski-Feder et al. 2000). This may reflect a poor representation of the distal telomeric regions within painting probes. In this case, the use of subtelomere probes (National Institutes of Health and Institute of Molecular Medicine Collaboration 1996) should have a better resolution. Second, color blending remains in the form of additional visible bands either at translocation breakpoints or at sites where chromosomes overlap. Third, the modified procedure will still miss small intrachromosomal rearrangements, such as small inversions, deletions, or amplifications. As previously emphasized by Uhrig et al. (1999), other strategies, such as multicolor bar coding, are superior for the analysis of intrachromosomal rearrangements.

Acknowledgments

The present study was supported by the Deutsche Krebshilfe grant 10-1392-Pa 1 and by the Deutsche Forschungsgemeinschaft grant Sp 460/3-1. J.A.'s stay in Munich was funded by the Deutscher Akademischer Austauschdienst and the University of Costa Rica. We thank Dr. Ferguson-Smith (Cambridge, England), for the chromosome painting probes, and Dr. Iver Petersen (Berlin), for cell line A427. We are grateful to Drs. Jan Murken and Heide Seidel, for providing the clinical samples. We thank Dr. Antje Wirtz, for critical reading of the manuscript and for numerous fruitful discussions.

References

Eils R, Uhrig S, Saracoglu K, Sätzler K, Bolzer A, Chassery JM, Ganser M, et al (1998) An optimized, fully automated system for fast and accurate identification of chromosomal rearrangements by multiplex-FISH (M-FISH). Cytogenet Cell Genet 82:160–171

Haddad BR, Schröck E, Meck J, Cowan J, Young H, Ferguson-

Smith MA, du Manoir S, et al (1998) Identification of de novo chromosomal markers and derivatives by spectral karyotyping. Hum Genet 103:619–625

Holinski-Feder E, Reyniers E, Uhrig S, Golla A, Wauters J, Kroisel P, Bossyut P, et al (2000) Familial mental retardation syndrome ATR-16 due to an inherited cryptic subtelomeric translocation, t(3;16)(q29;p13.3). Am J Hum Genet 66: 16–25

Macville M, Schröck E, Padilla-Nash H, Keck C, Ghadimi BM, Zimonjic D, Popescu N, et al (1999) Comprehensive and definitive molecular cytogenetic characterization of HeLa cells by spectral karyotyping. Cancer Res 59:141–150

National Institutes of Health and Institute of Molecular Medicine Collaboration (1996) A complete set of human telomeric probes and their clinical applications. Nat Genet 14: 86–89

Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, et al (1996) Multicolor spectral karyotyping of human chromosomes. Science 273: 494–497

Schröck E, Veldman T, Padilla-Nash H, Ning Y, Spurbeck J, Jalal S, Shaffer LG, et al (1997) Spectral karyotyping refines cytogenetic diagnostics of constitutional chromosomal abnormalities. Hum Genet 101:255–262

Speicher MR, Gwyn Ballard S, Ward DC (1996) Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet 12:368–375

Uhrig S, Schuffenhauer S, Fauth C, Wirtz A, Daumer-Haas C, Apacik C, Cohen M, et al (1999) Multiplex-FISH (M-FISH) for pre- and postnatal diagnostic applications. Am J Hum Genet 65:448–462