

# High resolution multicolor-banding: a new technique for refined FISH analysis of human chromosomes

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**Abstract.** A new multicolor-banding technique has been developed which allows the differentiation of chromosome region specific areas at the band level. This technique is based on the use of differently labeled overlapping microdissection

libraries. The changing fluorescence intensity ratios along the chromosomes are used to assign different pseudo-colors to specific chromosome regions. The multicolor banding of human chromosome 5 is presented as an example.

Genetic diseases are often due to numerical and structural chromosomal aberrations. In clinical cytogenetics analysis of the chromosomes is carried out on banded metaphase spreads. The application of banding techniques leads to a highly reproducible banding pattern which allows the reliable identification of every single chromosome and the assessment of structural aberrations. However, in numerous cases classical banding analysis does not provide sufficient information to detect subtle rearrangements.

Molecular cytogenetics was mainly established by improved fluorescence in situ hybridization techniques (FISH) (Langer et al., 1981; Pinkel et al., 1986) which in the meantime allow the identification of specific loci, specific chromosome regions and whole chromosomes at the DNA level independently of any banding pattern. Simultaneous detection of more than three target sequences using FISH was first presented by Nederlof (Nederlof et al., 1990). As a consequence of this principle, the separation of all 24 different human chromosomes via multiplex fluorescence in situ hybridization (M-FISH) (Speicher et al., 1996, Eils et al., 1998) and spectral karyotyping (SKY) (Schröck et al., 1996) has been established. Since these first

descriptions the usefulness of SKY has been demonstrated for the analyses of constitutional aberrations as well as tumor chromosomes (Schröck et al., 1997; Veldman et al., 1997; Rao et al., 1998). However, these methods cannot detect pericentric and paracentric inversions and precisely identify chromosome breakpoints. To overcome these limitations a more sophisticated banding technique is required.

For this purpose we developed a high resolution multicolor-banding method which has been applied to human chromosome 5 as an example.

## Methods and materials

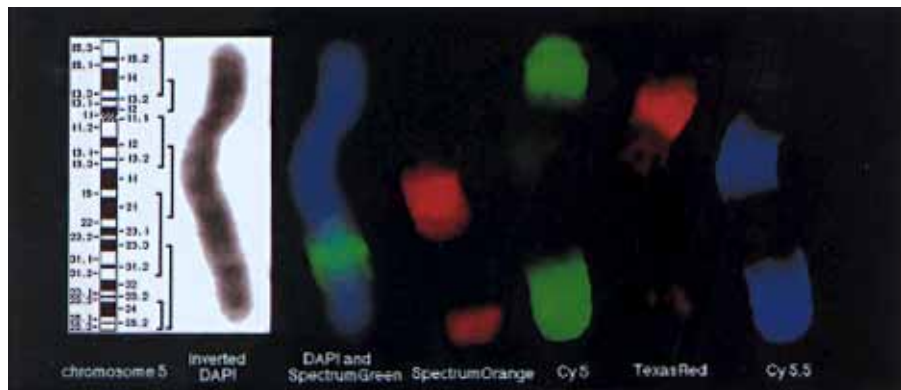
Region-specific partial chromosome paints (RPCP) were generated by microdissection (Lüdecke et al., 1989; Senger et al., 1990). For each region-specific library eight to ten chromosome fragments were excised. The respective regions were isolated with extended glass needles and the DNA was amplified by DOP-PCR (Telenius et al., 1992; Zhang et al., 1993; Chudoba et al., 1996; Senger et al., 1997). Altogether, seven overlapping microdissection DNA libraries of chromosome 5 were constructed, two within the p arm and five within the q arm. Each single chromosome region-specific PCP overlaps partly with the neighbouring one as shown in Fig. 1. The exact chromosome location of each library was assessed by reverse painting to normal prometaphase spreads. A banding pattern on the target chromosomes was generated by incorporation of BrdU in late S phase after synchronization and detection with an antiBrdU antibody coupled to FITC (Senger et al., 1993).

Five different fluorochromes were used to label the eight different DNA-libraries of which three were directly coupled to nucleotides (Spectrum-

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**Fig. 1.** Localization of the chromosome 5 region-specific partial chromosome paints (RPCPs) and pictures of the fluorescence signals taken with the individual filter combinations.



Green- and SpectrumOrange-dUTP, Vysis, and Texas Red-dUTP, Molecular Probes). Two further fluorochromes were used indirectly via biotin-dUTP detected with avidinCy5 (Amersham) and via digoxigenin-dUTP, Boehringer, Mannheim) detected with a mouse antidigoxigenin antibody (Boehringer, Mannheim) coupled to Cy5.5 (Mab-labeling kit, Amersham). Signal amplification was achieved with biotinylated antiavidin and digoxigenin-labeled anti-mouse FAb, respectively. Labeling of the DNA libraries was carried out in a way, that each RPCP as well as the overlapping regions are represented by a different fluorochrome or a unique combination of them (see Table 1).

Hybridization, post-hybridization washes and signal detection of the 24 color mix as well as of the RPCPs was carried out following standard protocols (Senger et al., 1993).

Microscopic analysis was performed using an Axioplan II microscope (Carl Zeiss, Jena GmbH,) equipped with an HBO 100 mercury lamp and filter sets for DAPI, FITC, Cy3, Cy3.5, Cy5 and Cy5.5 (Chroma Technology). Images were captured and processed using the isis/mFISH imaging system (MetaSystems GmbH, Altussheim, Germany). The software controls the motorized filter revolver as well as the excitation and emission filter wheels, thus automating the capture process completely.

Four cases were chosen in order to demonstrate the potential of the approach presented here for clarifying breakpoints involved in structural chromosome aberrations. Two cases with acute myeloid leukemia showed complex aberrations with an obvious deletion in one chromosome 5 in each case (Fig. 2B and C). The third case is a lymphoma with complex chromosome aberrations with involvement of chromosome 5 (Fig. 2D) and the fourth case is a constitutional paracentric inversion of chromosome 5 (Fig. 2E).

## Results and discussion

The different dissected copies within one RPCP are not absolutely identical and hence, there is a gradual decrease of the fluorescence intensities towards both ends of each library. Consequently, within overlapping regions the fluorescence signal intensity ratios change continuously along the longitudinal axis of the chromosome (see Fig. 1). The quantification of the intensity ratios along the chromosome and assignment of pseudo-colors to chromosome segments of similar intensity ratios provides the basis for high resolution color banding.

Image analyses of the multicolor labeled chromosome 5 have been performed with the mFISH extension of MetaSystems isis software (MetaSystems GmbH, Altussheim, Germany). A special function automatically divides the chromosomes into a predefined number of segments, where the length of each segment depends on the local variation of color information.

**Table 1.** Scheme for the labeling pattern of the region-specific partial chromosome paint

No of RPCP <sup>a</sup>	Fluorochrome <sup>b</sup>				
	Cy5	TR	Cy5.5	SO	SG
1	■				
1/2	□				
2		■			
2/3		□	□		
3			■		
3/4			□	□	
4				■	
4/5				□	□
5					■
5/6	□		□		□
6	■		■		
6/7	□		□	□	
7				■	

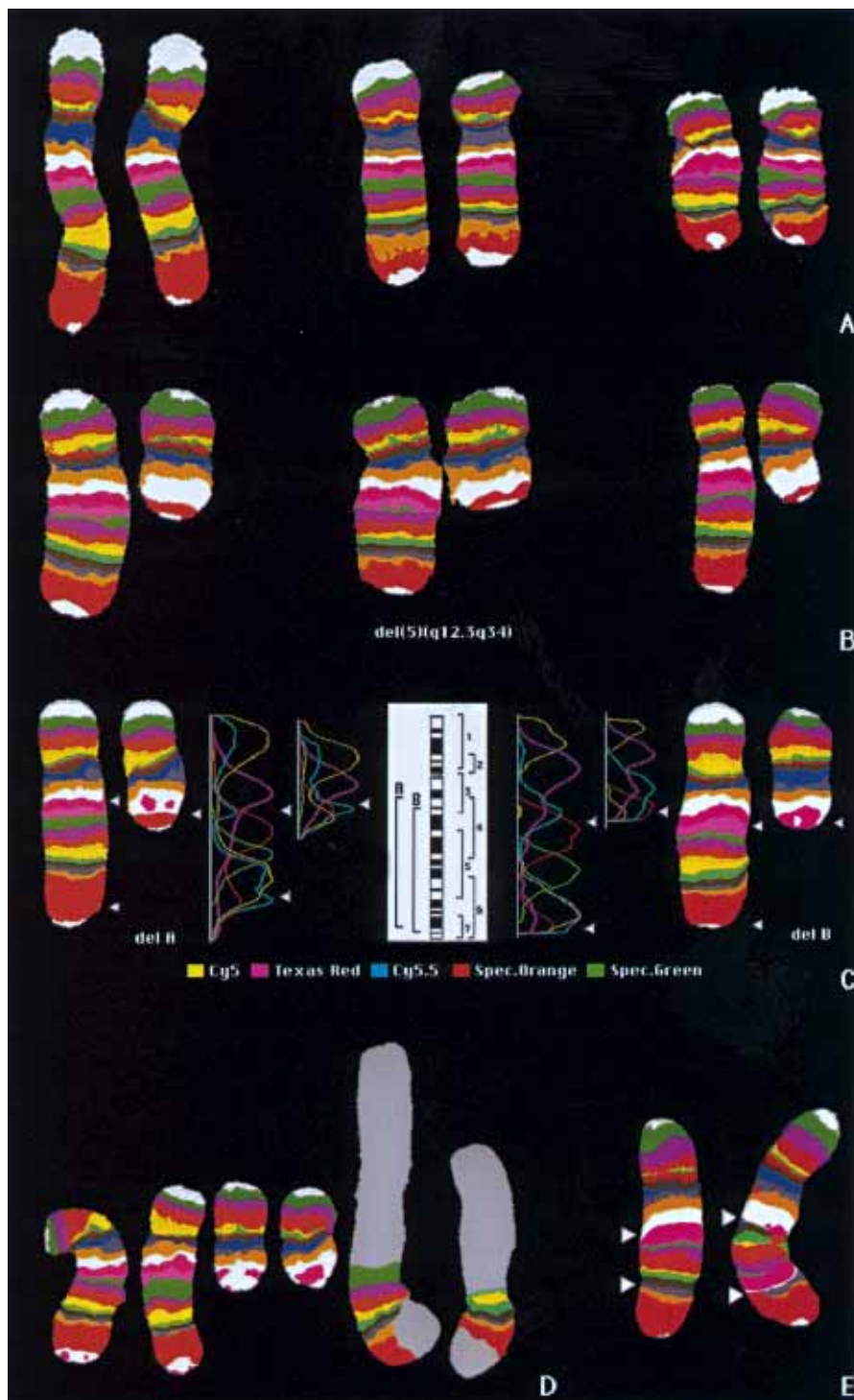
<sup>a</sup> RPCP = region-specific partial chromosome paint.

<sup>b</sup> TR = Texas Red; SO = Spectrum Orange; SG = Spectrum Green; ■ = labeling of the the RPCP with corresponding fluorochrome; □ = resulting labeling in the overlapping regions.

Regions of slowly varying fluorescence ratio are divided into longer segments compared to overlapping regions of individual RPCPs which exhibit an increased intensity ratio change along the chromosome axis. The color composition of each segment is quantitated using the multicolor analysis of isis/mFISH. Based on this analysis a pseudo-color can be assigned to each pixel resulting in the characteristic high resolution multicolor-banding pattern. At least 23 individual bands were obtained for chromosome 5 (see Fig. 2) comparable to the 500 band level for the whole chromosome complement.

Several chromosomes 5 at various condensation grades from different individuals have been compared after hybridization with the same probe set. In all instances the same multicolor-banding pattern has been observed (Fig. 2A) independently of the length of the chromosomes and the resolution of the GTG-banding pattern. This holds true even for tumor chromosomes, where banding resolution is often poor due to highly contracted chromosomes. Figure 2B demonstrates once more the reproducibility of the banding pattern. Here three examples

**Fig. 2.** Three examples presenting the analytical capabilities of high resolution multicolor-banding. **(A)** Three pairs of multicolor-banded normal chromosomes 5 at different lengths from normal lymphocyte metaphase spreads. **(B)** Three pairs of chromosomes 5 with a deletion of the long arm of one chromosome from a case with acute myeloid leukemia (AML). The normal chromosomes 5 show an identical multicolor banding pattern as well as the deleted ones. **(C)** Multicolor-banded chromosomes 5 from a metaphase spread of a patient with AML (left). One chromosome 5 shows an interstitial deletion of the long arm,  $\text{del}(5)(\text{q}13.1\text{q}34)$ . The second case (right) shows an interstitial deletion as well, but the breakpoints are slightly different,  $\text{del}(5)(\text{q}13.3\text{q}35.2)$ , which is undetectable by conventional cytogenetics. In the middle the ideogram of chromosome 5 is shown with the location of the region-specific paints indicated by brackets on the right-hand side. The brackets on the left-hand side represent the extension of the two deletions. The profiles on both sides of the ideogram show the fluorescence intensities of the five fluorochromes along the longitudinal axis of the respective chromosomes. **(D)** Chromosomes 5 and its derivatives from a case with a lymphoma. The tumor cells are characterized by a tetraploid cell clone, which shows two normal chromosomes 5, two deleted chromosomes 5 and two chromosomes 2 with chromosome 5 material inserted. However, the insertions are not identical. One insertion comprises exactly the chromosome region deleted in the  $\text{del}(5)(\text{q}13.3\text{q}35.2)$ , whereas the other insertion is smaller, ranging from  $5\text{q}23 \rightarrow \text{q}35.2$ . Altogether, this chromosome rearrangement results in a partial deletion of  $5\text{q}13.3 \rightarrow \text{q}22$ . By conventional cytogenetics alone it was not possible to characterize this complex chromosome rearrangement in detail. The insertion of chromosome 5 material into chromosome 2 was detected by FISH using a whole chromosome painting probe specific for chromosome 5 but only by applying high resolution multicolor-banding it was possible to describe the chromosome 5 rearrangements in full detail. **(E)** A paracentric inversion of chromosome 5 is shown. Conventional Giemsa-banding (not shown) identified the following breakpoints:  $5\text{q}14$  and  $5\text{q}32$ . However, after multicolor-banding analysis the breakpoints were corrected to  $\text{inv}(5)(\text{q}13.1\text{q}31.2)$ .



of chromosome 5 from different metaphase spreads from a case with a deletion within the long arm of one chromosome 5 are presented. The normal chromosomes 5 show an identical banding pattern and so do the deleted ones. Variations in the length of identical bands on different chromosomes are due to the different condensation behavior of light and dark G-bands (Hlisc et al., 1997). Dark G-bands split up in different bands on longer

chromosomes, whereas light G-bands remain rather unchanged. A similar effect is observed on the multicolor-banded chromosomes. For example the yellow band on the long arm of the left chromosome 5 in Fig. 2A is twice as large as the neighboring orange one. With increasing condensation the yellow band becomes smaller, whereas the orange one retains more or less the same size.

Definition of the color bands corresponding to the ISCN (1995) was carried out by comparing the multicolor banding pattern with the DAPI-banding and the fluorescence intensity profiles. The chromosome location of the microdissection libraries corresponds to the extent of the fluorescence intensity profiles along the chromosome and hence, chromosome aberrations result in changes of the multicolor banding pattern as well as changed profiles.

Figure 2C shows the results of multicolor-banding applied to different tumor chromosomes 5. Two cases with deleted chromosomes 5 are presented. The deletion ranges from 5q13.1 → q34 in one case (del A) and from 5q13.3 → q35.2 in the other case (del B). These differences in the localization of the breakpoints were not detectable using conventional cytogenetics, i.e. GTG banding on these kind of chromosomes, and could only be specified after high resolution multicolor banding analysis. A further example is presented in Fig. 2D where multicolor-banded chromosomes 5 are shown in a case with a lymphoma. The malignant cell clone was characterized by tetraploid metaphase spreads. Conventional cytogenetics detected two normal and two deleted chromosomes 5 together with further complex rearrangements. By applying chromosome 5 specific whole chromosome painting probes two derivative chromosomes 2 with chromosome 5 material inserted were identified, but it was impossible to describe the involved breakpoints in more detail. High resolution multicolor banding revealed the details of the cytogenetic aberrations regarding chromosome 5 (see Fig. 2D). A further example for the reliability of the high resolution multicolor-banding is presented in Fig. 2E. A paracentric inversion described as inv(5)(q14q32) after conventional Giemsa-banding was investigated and the breakpoints had to be corrected to inv(5)(q13.1q31.2) after multicolor-banding analysis.

A similar effect as already described by Speicher et al. (1996) due to the flaring of fluorescence signals was observed in the case with the insertion (Fig. 2D). The chromosome 5 specific probe set was hybridized together with a chromosome 2 specific painting probe and at the junction of the two chromosomes an irregular green band appeared. The effect seems to be

more important when indirect labeling is used, nevertheless, this special feature has to be carefully considered when aberrant multicolor banding patterns are interpreted.

The method presented here is an alternative to earlier published approaches of in situ hybridization based banding techniques like chromosome bar codes (Lengauer et al., 1993; Müller et al., 1997) and cross-species chromosome painting (Ferguson-Smith, 1997, Müller et al., 1998). The chromosome bar codes were generated using either YAC clones or Alu-PCR products from fragment hybrids. However, only a poor banding resolution was achieved and the probe sets used did not cover the entire chromosomes. FISH using probes from two different gibbon species results in a color banding pattern covering all human chromosomes, but only 81 segments were achieved (Müller et al., 1998) which is far below the banding resolution that could be realized in the high resolution multicolor banding technique presented here.

Our multicolor banding approach presented here effectively multiplies the initial probe kit resolution of 7 libraries by at least a factor of 3 to achieve high banding resolution with a minimum of libraries and, consequently a minimum of fluorochromes or fluorochrome combinations.

In conclusion, the new method of high resolution multicolor-banding has been demonstrated to open a new dimension in the characterization of chromosome aberrations with a high reproducibility, as evidenced in four examples (see Fig. 2). It provides great potential for a highly specific diagnosis, in particular in tumor cytogenetics. Here, multicolor banding can contribute to a more refined determination of breakpoints involved in chromosome aberrations because of the high resolution of this banding technique which is independent of chromosome contraction.

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