

Multicolor spectral karyotyping of rat chromosomes

A. Buwe,^a C. Steinlein,^a M.R. Koehler,^b I. Bar-Am,^c N. Katzin^c and M. Schmid^a

^aDepartment of Human Genetics, University of Würzburg, Biozentrum, Würzburg (Germany); ^bApplied Spectral Imaging, Mannheim (Germany); ^cApplied Spectral Imaging, Migdal Ha'Eemek (Israel)

Abstract. Rat and mouse have become important animal models to study various human diseases such as cancer. Cytogenetic analysis of the respective karyotypes is frequently required to investigate the causative genetic defects and especially neoplastic cells often show complex chromosome aberrations and many different marker chromosomes. However, structural homogeneity of the chromosomes in these species as well as less pronounced differences in banding patterns make it difficult to assign genetic abnormalities to certain chromosomes by conventional banding techniques. Here we report for the first time the successful application of multicolor spectral karyotyping (SKY) to rat chromosomes, which allows unequivocal identification of all rat chromosomes with the exception of chromosomes 13 and 14 in different colors, thus enabling the elucidation of even complex rearrangements in the rat karyotype. Flow-sorted chromosome specific painting probes for all

22 rat chromosomes (20 autosomes, X, and Y) were combinatorially labeled by a set of five different fluorochromes and hybridized in situ to metaphase spreads of a healthy rat, to diakinesis from testicular material, and to cells from a rat FAO hepatoma cell line. Measuring the complete spectrum at each image point by using the SpectraCube[®] spectral imaging system and respective computer software allowed identification of the individual rat chromosomes by their specific emission spectra. Classification algorithms in the analysis software can then display the rat chromosomes in specific pseudo-colors and automatically order them in a karyotype table. After its successful application to human and mouse chromosomes, spectral karyotyping of rat chromosomes now also allows cytogenetic screening of the complete rat genome by a single hybridization.

Copyright © 2003 S. Karger AG, Basel

Human diseases are often both, polygenic and heterogeneous, are influenced by many environmental factors, and the heterogeneous human genetic constitution further contributes to the complexity of the situation. An elegant way to reduce the influences of such complicating factors is the use of animal model systems to study the underlying defects in human diseases. The rat is widely used as a model for experimental studies (Gill et al., 1989; Jacob et al., 1995). Many research groups have created

databases providing a solid basis for detailed comparative genetic analysis of rat, mouse and human genomes, i.e. to investigate and identify genetic aberrations involved in the various steps leading to oncogenic transformation (The Jackson Laboratory, Mouse Genome Informatics: <http://mgd.niaiaffrc.go.jp/>; The Genome Database: <http://gdbwww.gdb.org/>; RATMAP: The rat Genome Database: <http://ratmap.gen.gu.se/>; The ARB Rat Genetic Database: <http://www.nih.gov/niams/scientific/ratgbase/>).

Translocations, aneuploidy and marker chromosomes are frequently observed in cancer cells. However, limitations in conventional banding techniques as well as the often complex nature of cancer-related chromosome aberrations render it very difficult to identify the chromosomes involved in such aberrations. This especially applies to species like mouse and rat in which the less pronounced differences in banding patterns strongly impair the analysis of complex rearrangements by conventional chromosome banding. Fluorescence in situ hybridi-

Received 24 July 2003; revision accepted 2 September 2003.

Request reprints from Dr. M. Schmid

Department of Human Genetics, University of Würzburg
Biozentrum, Am Hubland, D-97074 Würzburg (Germany)
telephone: +49-931-888-4077; fax: +49-931-888-4058
e-mail: m.schmid@biozentrum.uni-wuerzburg.de

Table 1. Fluorescence labeling scheme of rat chromosomes

| Dye ^a | Chromosome | | | | | | | | | | | | | | | | | | | | | |
|------------------|------------|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | X | Y |
| A | + | | | + | | + | | | + | | | | | | | + | + | + | | + | + | |
| B | | + | | | + | + | | | + | + | + | | | | + | | | | | | | |
| C | | | + | + | | + | | | | + | + | + | + | + | | | | | + | | | + |
| D | | | + | | | | | + | | + | | + | | | | + | | + | + | + | | + |
| E | + | | + | | + | | + | | | | | | + | + | + | | | | | + | + | + |

^a A = Rhodamine (orange); B = Texas-Red (red); C = Cy5 (infrared 1); D = FITC (green); E = Cy5.5 (infrared 2)

zation (FISH) offers a higher sensitivity, specificity and resolution for delineating chromosome aberrations than banding analysis. However, the inability of the FISH technique to screen the whole genome in one experiment has restricted its use for a long time to the confirmation of already existing suspects by the use of specific probes for the respective chromosomes or chromosome regions. The recent introduction of multicolor FISH techniques in human cytogenetics (Schröck et al., 1996; Speicher et al., 1996) could overcome these limitations by providing the ability to discriminate all 24 human chromosomes in different colors.

Here we apply the spectral karyotyping (SKY) technique originally developed for the multicolor differentiation of human and mouse chromosomes (Liyanage et al., 1996; Schröck et al., 1996) to the rat karyotype. In this case the spectral imaging technology was used to measure chromosome-specific emission spectra generated by combinatorially labeled chromosome-specific painting probes hybridized in situ to metaphase spreads of the rat. Based on the obtained discrete emission spectra at all pixels of the CCD-image, the spectral information is converted by spectral classification algorithms (adapted to the rat) resulting in the assignment of a unique pseudo-color to all sample points having identical spectra. This allows the unequivocal identification of the rat chromosomes in different colors and the karyotype is arranged automatically according to the nomenclature rules for G-bands in rat chromosomes (Levan, 1974).

Materials and methods

Preparation of rat metaphase chromosomes

Mitotic chromosomes were obtained directly from bone marrow after in vivo treatment with 0.03% colchicine according to standard techniques. Male meiotic chromosomes were prepared following the method of Evans et al. (1964). After hypotonic treatment with 0.075 M KCl for about 30 min, the cells were fixed in methanol:glacial acetic acid (3:1). The suspension was dropped onto cleaned wet slides and air dried. Prior to hybridization slides were pretreated with 1% formaldehyde and then dehydrated through an alcohol series. For all hybridizations freshly prepared slides were used.

Labeling of flow-sorted rat chromosome-specific painting probes

Flow-sorted rat chromosome-specific painting probes were kindly obtained from P. O'Brien and M. Ferguson-Smith, Cambridge, UK. Fluorescent labeling of the painting probes was achieved by DOP-PCR using five different dyes (FITC: green; Rhodamine: orange; Texas-Red: red; Cy5: infrared 1; and Cy5.5: infrared 2) and combinations thereof (Table 1). Flow-sorting did not allow to differentiate rat chromosomes 13, 14, and 15 as well as chromosomes 11 and 15. To resolve this problem the mixture of chromosomes 13, 14, and 15 was combinatorially labeled by the dye combination

C/E, and for the mixture of chromosomes 11 and 15 a dye combination B/C was used (Table 1). This results in unique combinations for chromosomes 11 (B/C) and 15 (B/C/E). Thus, only chromosomes 13 and 14 share the same dye combination leaving them indistinguishable by their emission spectra. However, this is compensated to a certain extent by their marked morphological differences.

In situ hybridization and detection

Denaturation of probe cocktail and chromosome spreads, in situ hybridization, as well as washing and detection was carried out according to the manufacturer's instructions (Applied Spectral Imaging, Israel). Briefly, the rat SKY probe cocktail was denatured for 7 min at 80 °C, followed by pre-annealing for 1 h at 37 °C. Slides were denatured in 70% formamide, 2× SSC for 90 s at 70 °C. For hybridization 3.5 µl probe cocktail was applied to denatured metaphase preparations and covered by an 18 × 18 mm cover slip. After hybridization at 37 °C for 2 days, slides were washed and haptenized probe sequences were detected following the manufacturer's protocol. Slides were counterstained with DAPI and embedded in an antifade reagent (para-phenylenediamine).

Spectral imaging

Fluorescent probe signals were visualized using a 63× oil immersion objective on a Zeiss Axiophot microscope equipped for epifluorescence. For dye excitation the light of a 75 W Xenon lamp was directed through a custom designed SKY filter set (Chroma Technology, Brattleboro, USA). Spectral images were acquired using an SD200-H spectral bioimaging system (Applied Spectral Imaging, Israel) and analyzed by the respective software package SkyView Vers. 1.6.

Results and discussion

In this study we report for the first time the successful extension of multicolor spectral karyotyping (SKY) to the rat karyotype. Flow-sorted whole chromosome painting probes of the rat were combinatorially labeled to obtain unique dye combinations for 20 of the 22 rat chromosomes and were hybridized in situ to mitotic metaphase spreads of a normal male rat (Fig. 1). The complex chromosome-specific emission spectra resulting from the combinatorial labeling were measured by a spectral imaging system which simultaneously records in a single exposure the spectra at all pixels of a CCD image. The analysis software first shows the image as a Red-Green-Blue (RGB) display (Figs. 1a, 2a, 3a) to visualize also the infrared dyes which otherwise would not be seen by the human eye. Displaying the pixels with identical spectra in unique pseudo-colors therefore displays each of the chromosomes in a different color allowing unequivocal (per pixel) chromosome identification and accurate automatic karyotyping. Although flow-sorting of the rat chromosomes could not separate chromosomes 13, 14, and 15 as well as 11 and 15 from each other, the different combinato-

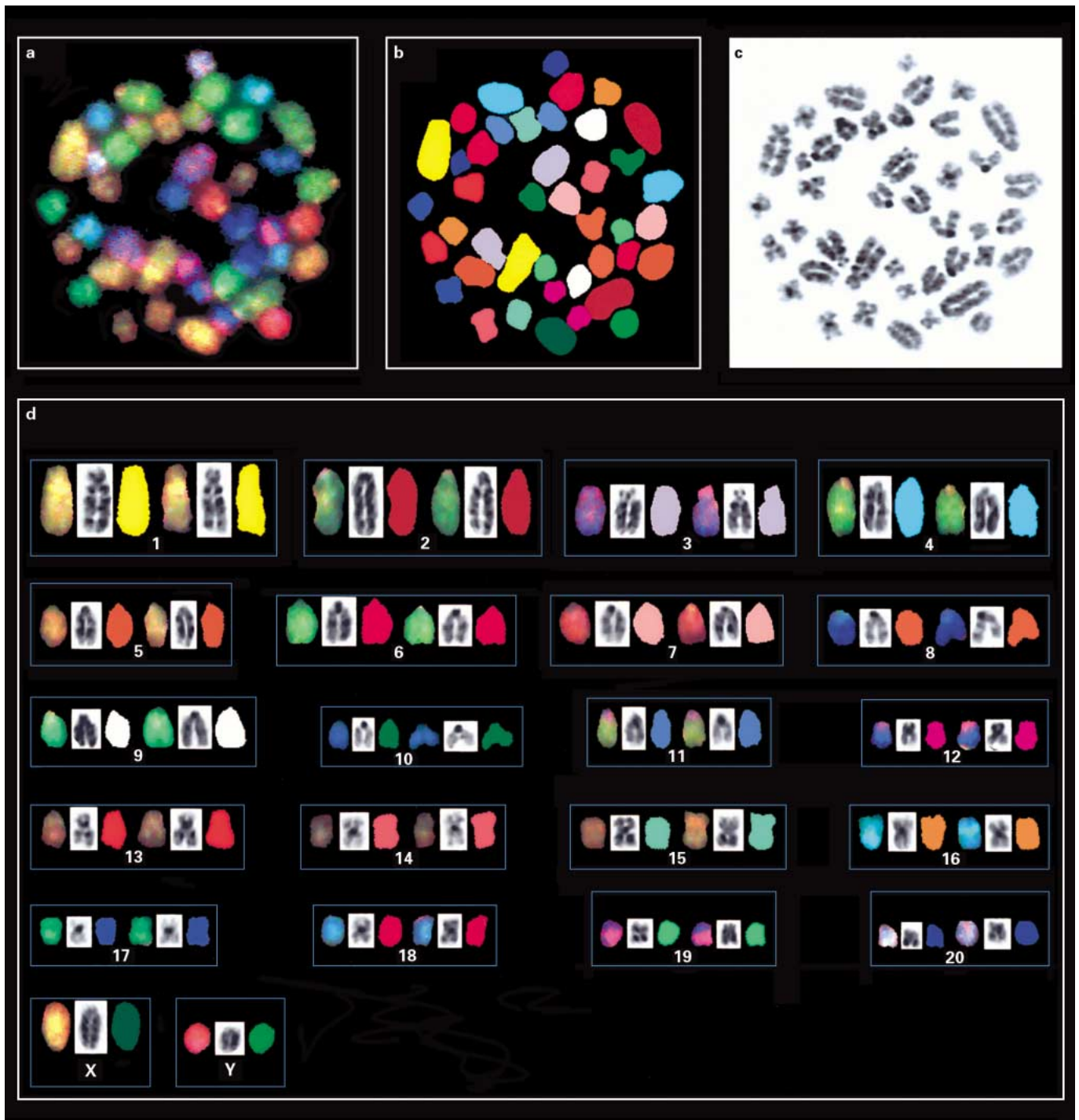


Fig. 1. Spectral karyotyping of a mitotic rat metaphase. **(a)** RGB display after hybridization of a normal male metaphase with the SKY probe cocktail. **(b)** Classified pseudo-color image of the same metaphase after per-pixel classification of the spectral data. **(c)** Inverted DAPI-stained image. **(d)** Karyotype of the metaphase showing RGB color (left), inverted DAPI-stained chromosomes (middle) and spectrally classified, pseudo-colored chromosomes (right).

rial labeling schemes of these two mixtures led to a unique dye combination for chromosomes 11 and 15 as a result of the hybridization process (compare Table 1). Thus, only chromosomes 13 and 14 have identical spectra which cannot be differentiated by the spectral measurement. This is also reflected in a similar RGB color of chromosomes 13 and 14 in the RGB display

of the images (Figs. 1a, d, 2a, d). However, marked differences in the G-banding pattern between these two chromosomes are visible in the inverted DAPI counterstain (Figs. 1c, 2c, 3c) which allows to correct misclassifications of chromosomes 13 and 14. It is also noticeable that several chromosomes show different colors in their centromeric region compared to



Fig. 2. Spectral karyotyping of a rat diakinesis. **(a)** RGB display after SKY hybridization. **(b)** Classified pseudo-color image of the same diakinesis after per-pixel classification of the spectral data. **(c)** Inverted DAPI-stained image. **(d)** Karyotype of the diakinesis showing RGB color (left), inverted DAPI-stained chromosomes (middle) and spectrally classified, pseudo-colored chromosomes (right).

their euchromatic part (Fig. 1d, chromosomes 3, 4, and 11). This phenomenon is caused by cross-hybridization of centromeric heterochromatin, which could not sufficiently be suppressed during preannealing and hybridization steps. As a consequence chromosome-specific spectra at the points of cross-hybridization are altered making the classification process ambiguous. Therefore, these areas should be excluded from analysis (Lee et al., 2001). SKY analysis is not restricted to study mitotic metaphase spreads, but can also be applied to investigate meiotic chromosomes. Figure 2 represents a diakinesis of a meiotic cell division originating from the rat used for mitotic chromosome analysis. The multicolor karyotyping analysis immediately identifies the sex chromosomes (Fig. 2a, b, d) and it

can be noticed that all autosomal bivalents show a ring-like arrangement, whereas the gonosomes show an end-to-end-association (Fig. 2c, d).

Especially in tumor cells multiple and complex aberrations are found which often cannot, or only insufficiently be detected and interpreted by conventional banding techniques. To demonstrate the potential and usefulness of the multicolor spectral karyotyping approach for rat chromosomes we have hybridized a FAO hepatoma cell line (Petzinger et al., 1994) of the rat showing a large number of chromosome aberrations which are difficult to identify by G-banding. Figure 3 displays a representative metaphase of this cell line. The average chromosome number was determined to be 49 (ranging from 43 to 55 chro-

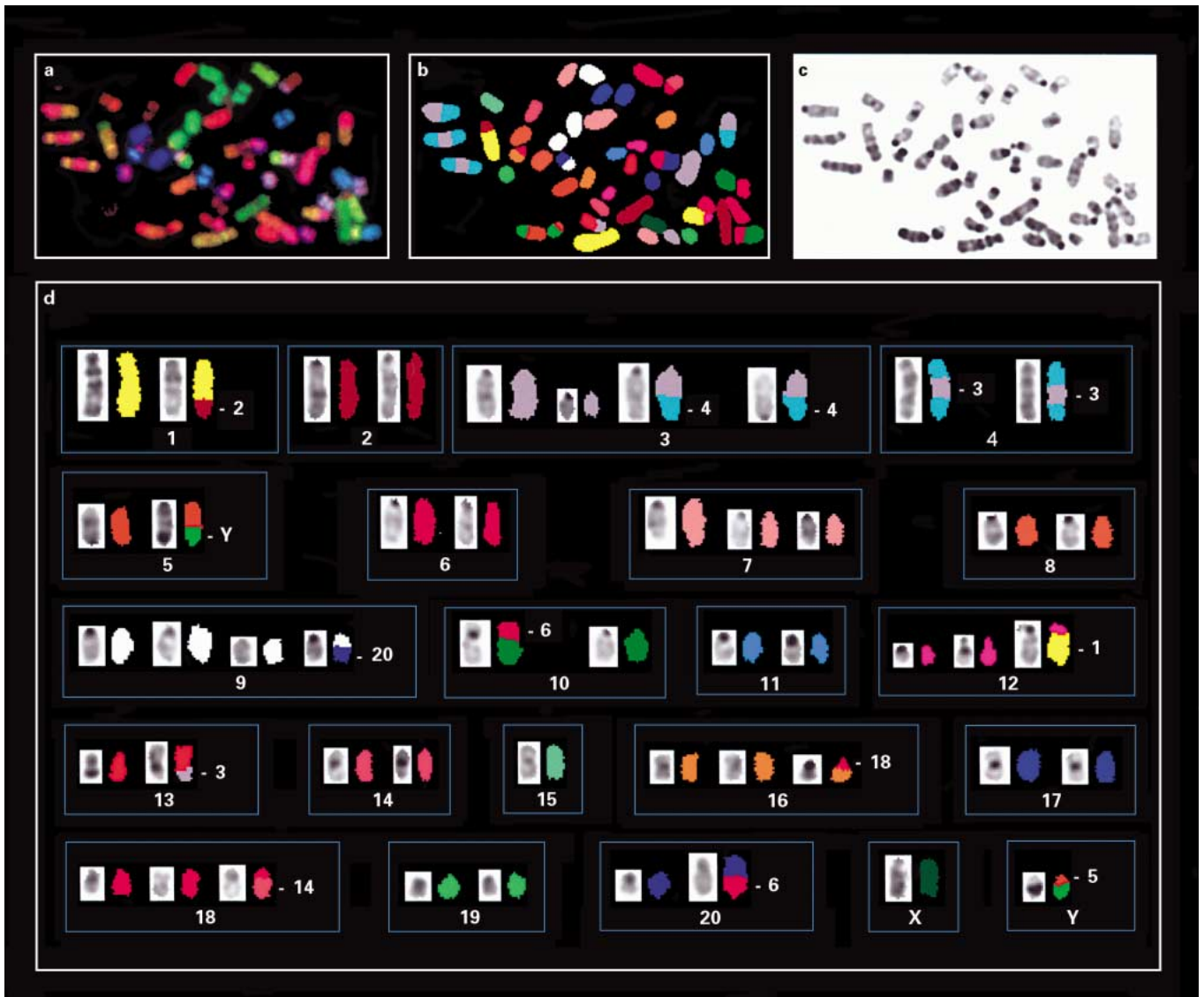


Fig. 3. Spectral karyotyping of a FAO hepatoma cell line metaphase. **(a)** RGB display after SKY hybridization. **(b)** Classified pseudo-color image of the same metaphase after per-pixel classification of the spectral data. **(c)** Inverted DAPI-stained image. **(d)** Karyotype of the metaphase showing inverted DAPI-stained (left) and spectrally classified, pseudo-colored chromosomes (right).

mosomes in 20 metaphases analyzed). SKY analysis readily reveals the translocations present in this tumor cell line, the following aberrations being observed most frequently: der(Y)t(Y;5), der(1)t(1;2), der(3)t(3;4), der(4)t(4;3;4), der(9)t(9;6;20), der(10)t(6;10), der(12)t(1;12), der(13)t(3;13), der(16)t(16;18), der(18)t(14;18), der(20)t(6;20).

Since its introduction in 1996 (Schröck et al., 1996) spectral karyotyping has been extensively used to elucidate aberrations of human chromosomes in clinical cases (e.g. Schroeck et al., 1997; Heng et al., 2003) and especially in oncological cases including patients with hematological (e.g. Rowley et al., 1999; Knutsen et al., 2003) as well as those with solid tumor diseases (e.g. Abdel-Rahman et al., 2001; Padilla-Nash et al., 2001). The results of these studies could contribute to diagnosis, therapy decisions, and follow-up studies in the respective cases and also

facilitated the identification and mapping of disease related candidate genes (e.g. Griffin et al., 1999; Chen et al., 2000). However, for a thorough understanding of the causative genetic factors leading to human diseases such as cancer, animal models are an important method to experimentally control and investigate candidate genes for their phenotypic effects and functions (e.g. transgenic animals, knock-outs, etc.). Mouse and rat are the most popular species used as model systems for human diseases, but the structural homogeneity of their chromosomes and rather poorly defined differences in banding patterns require skilled and experienced cytogeneticists to identify the chromosomes with certainty by traditional banding techniques. The applicability of multicolor karyotyping to mice chromosomes (Liyanage et al., 1996) has recently contributed substantially to the cytogenetic characterization of mouse mod-

el systems (e.g. Coleman et al., 1999; Difilippantonio et al., 2000; Guttenbach et al., 2001; Montagna et al., 2003).

With the availability of flow-sorted rat chromosome paints we could demonstrate the successful extension of spectral karyotyping to the rat genome. Similar to the situation in

mouse it can be expected that multicolor karyotyping will likewise greatly facilitate the identification of rat chromosomes and enhance the resolution, accuracy and speed in the genetic analysis of respective rat models of human diseases.

References

- Abdel-Rahman WM, Katsura K, Rens W, Gorman PA, Sheer D, Bicknell D, Bodmer WF, Arends MJ, Wyllie AH, Edwards PA: Spectral karyotyping suggests additional subsets of colorectal cancers characterized by pattern of chromosome rearrangement. *Proc natl Acad Sci, USA* 98:2538–2543 (2001).
- Chen Z, Coffin ZM, Scott S, Meloni-Ehrig A, Shepard R, Issa B, Forsyth DR, Sandberg AA, Brothman AR, Lowichik A: Evidence by spectral karyotyping that 8q11.2 is nonrandomly involved in lipoblastoma. *J molec Diag* 2:73–77 (2000).
- Coleman AE, Forest ST, McNeil N, Kovalchuk AL, Ried T, Janz S: Cytogenetic analysis of the bipotential murine pre-B cell lymphoma, P3388, and its derivative macrophage-like tumor, P388D1, using SKY and CGH. *Leukemia* 13:1592–1600 (1999).
- Difilippantonio MJ, Zhu J, Tang Chen T, Meffre E, Nussenzweig NC, Max EE, Ried T, Nussenzweig A: DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. *Nature* 404:510–514 (2000).
- Evans EP, Breckon G, Ford CE: An air-drying method for meiotic preparations from mammalian testes. *Cytogenet Cell Genet* 3:289–294 (1964).
- Gill TJ, Smith GJ, Wissler RW, Kunz HW: The rat as an experimental animal. *Science* 245:269–276 (1989).
- Griffin CA, Hawkins AL, Dvorak C, Henkle C, Ellingham T, Perlman EJ: Recurrent involvement of 2p23 in inflammatory myofibroblastic tumors. *Cancer Res* 59:2776–2780 (1999).
- Guttenbach M, Steinlein C, Engel W, Schmid M: Cytogenetic characterization of the TM4 mouse Sertoli cell line. I. Conventional banding techniques, FISH and SKY. *Cytogenet Cell Genet* 94:71–78 (2001).
- Heng HHQ, Ye CJ, Yang F, Ebrahim S, Liu G, Bremer SW, Thomas CM et al: Analysis of marker or complex chromosomal rearrangements present in pre- and post-natal karyotypes utilizing a combination of G-banding, spectral karyotyping and fluorescence in situ hybridization. *Clin Genet* 63:358–367 (2003).
- Jacob HJ, Brown DM, Bunker RK, Daly MJ, Dzau VJ, Goodman A, Koike G et al: A genetic linkage map of the laboratory rat, *Rattus norvegicus*. *Nature Genet* 9:63–69 (1995).
- Knutsen T, Pack S, Petropavlovskaja M, Padilla-Nash H, Knight C, Mickley LA, Ried T, Elwood PC, Roberts SJ: Cytogenetic, spectral karyotyping, fluorescence in situ hybridization, and comparative genomic hybridization characterization of two new secondary leukemia cell lines with 5q deletions, and MYC and MLL amplification. *Genes Chrom Cancer* 37:270–281 (2003).
- Lee C, Gisselsson D, Jin C, Nordgren A, Ferguson DO, Blennow E, Fletcher JA, Morton CC: Limitations of chromosome classification by multicolor karyotyping. *Am J hum Genet* 68:1043–1047 (2001).
- Levan G: Nomenclature for G-bands in rat chromosomes. *Hereditas* 77:37–52 (1974).
- Liyanage M, Coleman A, du Manoir S, Veldmann T, McCormack S, Dickson R, Barlow C et al: Multicolour spectral karyotyping of mouse chromosomes. *Nature Genet* 14:312–315 (1996).
- Montagna C, Lyu MS, Hunter K, Lukes L, Lowther W, Reppert T, Hissong B, Weaver Z, Ried T: The Septin 9 (MSF) gene is amplified and overexpressed in mouse mammary gland adenocarcinomas and human breast cancer cell lines. *Cancer Res* 63:2179–2187 (2003).
- Padilla-Nash HM, Heselmeyer-Haddad K, Wangsa D, Zhang H, Ghadimi BM, Macville M, Augustus M, Schröck E, Hilgenfeld E, Ried T: Jumping translocations are common in solid tumor cell lines and result in recurrent fusions of whole chromosome arms. *Genes Chrom Cancer* 30:349–363 (2001).
- Petzinger E, Follmann W, Blumrich M, Walther P, Hentschel J, Bette P, Maurice M, Feldmann G: Immortalization of rat hepatocytes by fusion with hepatoma cells. I. Cloning of a hepatocytoma cell line with bile canaliculi. *Eur J Cell Biol* 64:328–38 (1994).
- Rowley JD, Reshmi S, Carlson K, Roulston D: Spectral karyotype analysis of T-cell acute leukemia. *Blood* 93:2038–2042 (1999).
- Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y et al: Multicolor spectral karyotyping of human chromosomes. *Science* 273:494–497 (1996).
- Schroeck E, Veldman T, Padilla-Nash H, Ning Y, Spurbek J, Jalal S, Schaffer LG, Papenhausen P, Kozma C, Phelan MC, Kjeldsen E, Schonberg SA, Biesecker L, du Manoir S, Ried T: Spectral karyotyping refines cytogenetic diagnostics of constitutional chromosomal abnormalities. *Hum Genet* 101:255–262 (1997).
- Speicher MR, Ballard SG, Ward DC: Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genet* 12:368–375 (1996).