© 1996 Nature Publishing Group http://www.nature.com/naturegenetics

Karyotyping human chromosomes by combinatorial multi-fluor FISH

Michael R. Speicher, Stephen Gwyn Ballard & David C. Ward

We have developed epifluorescence filter sets and computer software for the detection and discrimination of 27 different DNA probes hybridized simultaneously. For karyotype analysis, a pool of human chromosome painting probes, each labelled with a different fluor combination, was hybridized to metaphase chromosomes prepared from normal cells, clinical specimens, and neoplastic cell lines. Both simple and complex chromosomal rearrangements could be detected rapidly and unequivocally; many of the more complex chromosomal abnormalities could not be delineated by conventional cytogenetic banding techniques. Our data suggest that multiplex-fluorescence *in situ* hybridization (M-FISH) could have wide clinical utility and complement standard cytogenetics, particularly for the characterization of complex karyotypes.

Chromosome karyotyping by conventional cytogenetic banding methods is both time consuming, expensive, and difficult to automate. The detection of recurring genetic changes in solid tumour tissues by karyotyping is particularly problematic because of the difficulty in routinely preparing metaphase spreads of sufficient quality and quantity and the complex nature of many of the chromosomal changes. Marker chromosome identification based solely on banding patterns is extremely difficult. Indeed, attempts to fully automate karyotype analysis over the past twenty years have failed because robust computer algorithms could not be developed to reliably decipher complex banding patterns, particularly those of extensively rearranged chromosomes.

Fluorescence *in situ* hybridization (FISH) is a powerful tool for the analysis of genes and chromosomes because of its high absolute sensitivity and its ability to provide information at the single gene/single cell level. For this reason FISH techniques are being increasingly used in both basic research and clinical applications[1,2]. One of the advantages of fluorescence for the detection of hybridization probes is that several targets can be visualized simultaneously in the same sample. Using either combinatorial[3–5] or ratio-labelling[6–8] strategies, many more targets can be discriminated than the number of spectrally resolvable fluorophores. The simultaneous visualization of seven different probes labelled combinatorially with up to three fluors and 12 different probes labelled with different fluor ratios has been reported[5–6,9–12]. Combinatorial labelling provides the simplest way to label probes in a multiplex fashion since a probe fluor is either completely absent (0) or present in unit amounts (1); image analysis is thus more amenable to automation, and a number of experimental artifacts are avoided (such as differential photobleaching of the fluors and the effects of changing excitation source power spectrum).

The number of useful boolean combinations of N fluors is 2^N-1. Thus, for a single fluor A, there is only one useful combination (A = 1), while for two fluors, A and B, there are three useful combinations (A = 1, B = 0; A = 0, B = 1; A = 1, B = 1). There are seven combinations for three fluors, 15 combinations for four fluors, 31 combinations for five fluors, 63 combinations for six fluors, and so on. To uniquely identify all 24 chromosome types in the human genome using chromosome painting probes, five spectrally distinguishable fluors are needed. Each probe is thereby provided with a distinct spectral signature dictated by its fluor composition.

We describe a set of six fluors and corresponding optical filters spaced across the spectral interval 350–770 nm that give a high degree of discrimination between all possible fluor pairs. Using these fluors and filter sets, we have demonstrated simultaneous visualization of 27 combinatorially labelled probes applied to chromosome karyotyping by FISH, including whole chromosome paints, chromosome arm or band specific probes, and YAC clones.

Departments of Genetics and Molecular Biophysics and Biochemistry, Yale University School of Medicine, 333 Cedar St., New Haven, Connecticut 06510, USA

Correspondence should be addressed to M.R.S.

Selection of fluors and high contrast filters

The limited spectral bandwidth available for fluorescence imaging (~350–800 nm), and the extensive overlap between the spectra of organic fluors, makes

© 1996 Nature Publishing Group http://www.nature.com/naturegenetics

Table 1 Epicube filter configuration for 75 W Xe arc source

	DAPI	FITC	Cy3	Cy3.5	Cy5	Cy7
Excitation filter	Zeiss 365 nm	Omega 455DF70	Omega 546DF10	Ealing 35-3763	Omega 640DF20	Omega 740DF25
Dichroic beamsplitter	Zeiss 395 nm	Omega 505DRLP02	Omega 560DRLP02	Omega 590DRLP02	Omega 645DRLP02	Omega 777DRLP02
Emission filter	Zeiss >397 nm	Omega 530DF30	Ealing 35-3722	Zeiss 630/30	Omega 670DF32	Omega 780EFLP
IR blocking	Schott BG38	Schott BG38	Schott BG38	Schott BG38	Oriel 58893	Oriel 58895

separating multiple fluors spectroscopically a significant technical challenge. Choice of the fluors and filters in Table 1 was based on computer modelling of their contrast parameters using digitized excitation and emission spectra of a number of common fluors, the transmittance spectra of interference filters and dichroic beamsplitters from several optical suppliers (Omega, Ealing, Oriel and Zeiss), and the power spectra of the high pressure mercury arc, high pressure xenon arc, the Hg-Xe arc, the quartz tungsten-halogen lamp and various laser excitation sources. Excitation and emission contrast ratios were computed for every fluor relative to its two nearest spectral neighbours. Filters were then selected to achieve >90% discrimination. This level of contrast is not generally achievable using either excitation selection or emission selection alone, no matter how narrow the filter bandwidths. Thus excitation selection and emission selection were applied simultaneously. Excitation contrast was favored whenever possible, to avoid unnecessary wasting of fluorescence photons (and hence unproductive photobleaching of the fluor).

The chosen fluor set consisted of 4'-6-diamidino-2-phenylindole (DAPI, a general DNA counterstain), fluorescein (FITC), and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. The absorption and emission maxima, respectively, for these fluors are: DAPI (350nm; 456nm), FITC (490nm; 520nm), Cy3 (554nm; 568nm), Cy3.5 (581nm; 588nm), Cy5 (652nm; 672nm), Cy5.5 (682nm; 703nm) and Cy7 (755nm; 778nm). The excitation and emission spectra, extinction coefficients and quantum yield of these fluors are described[13–16]. All fluors were excited with a 75W Xenon arc.

To attain the required selectivity, filters with bandwidths in the range of 5–15 nm (compared to approximately 50 nm or more for 'standard' filter sets) were required. Further, it was necessary to both excite and detect the fluors at wavelengths far from their spectral maxima. Emission bandwidths were made as wide as possible. For low-noise detectors such as the cooled CCD camera used here, restricting the excitation bandwidth has little effect on attainable signal to noise ratios; the only drawback is increased exposure time.

Epifluorescence filter cubes constructed using the bandpass and dichroic filters listed in Table 1 were tested experimentally for spectral contrast between adjacent fluors. Good contrast (>92%) was attained in practice for all fluors except the Cy5/Cy 5.5 pair, which was marginal (80–90%). For this reason, Cy 5.5 was omitted from the combinatorial labelling experiments described below. It is vital to prevent infra-red light emitted by the arc lamp from reaching the detector; CCD chips are extremely sensitive in this region. Thus, appropriate IR blocking filters (Table 1) were inserted in the image path immediately in front of the CCD window, to minimize loss of image quality.

Imaging software

Multi-fluor combinatorial labelling depends on acquiring and analysing the spectral signature of each probe, that is, the intensity values of each of the component fluors in the six imager channels. Critical features are accurate alignment of source images, correction of chromatic crosstalk, and quantitation of the intensity of each fluor. To circumvent manual image manipulation we developed the necessary software to achieve these functions (see Methods). This software carries out the following steps in sequential order: 1) correction of the geometric image displacements caused by wedging in the emission interference filters, and by mechanical noise; 2) calculation of a DAPI segmentation mask to delineate all of the chromosomes in a metaphase spread; 3) calculation and subtraction of the background intensity values for each fluor, calculation of a threshold value, and creation of a segmentation mask for each fluor; 4) utilization of the segmentation mask of each fluor to establish a 'boolean' spectral signature of each probe; 5) visualization of the material hybridizing to each chromosome probe next to the DAPI image to facilitate chromosome identification; 6) creation of a com-

Table 2 Combinatorial labelling scheme used for the simultaneous labelling of all 24 chromosomes

a, 24-colour experiment with microdissected whole chromosome painting probes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
Fluoroscein	x					x	x		x				x		x		x			x	x	x		x
Cy3			x		x		x		x			x	x			x			x				x	x
Cy3.5				x		x	x				x	x		x	x			x	x		x		x	
Cy5								x	x	x	x			x		x			x	x	x	x		
Cy7		x			x			x				x	x	x	x		x	x		x				

b, 27-colour experiment with microdissected whole chromosome painting probes and chromosome arm painting probes

	1	2	3p	3q	4	5p	5q	6	7	8	9	10	11p	11q	12	13	14	15	16	17	18	19	20	21	22	X	Y
Fluoroscein	x							x	x		x		x			x		x		x			x	x	x		x
Cy3			x	x		x	x		x		x				x	x			x			x				x	x
Cy3.5					x	x		x	x				x	x	x		x	x			x	x		x		x	
Cy5			x			x				x	x	x	x	x			x		x			x	x	x	x		
Cy7		x	x			x	x			x			x		x	x	x	x		x	x		x				

© 1996 Nature Publishing Group http://www.nature.com/naturegenetics

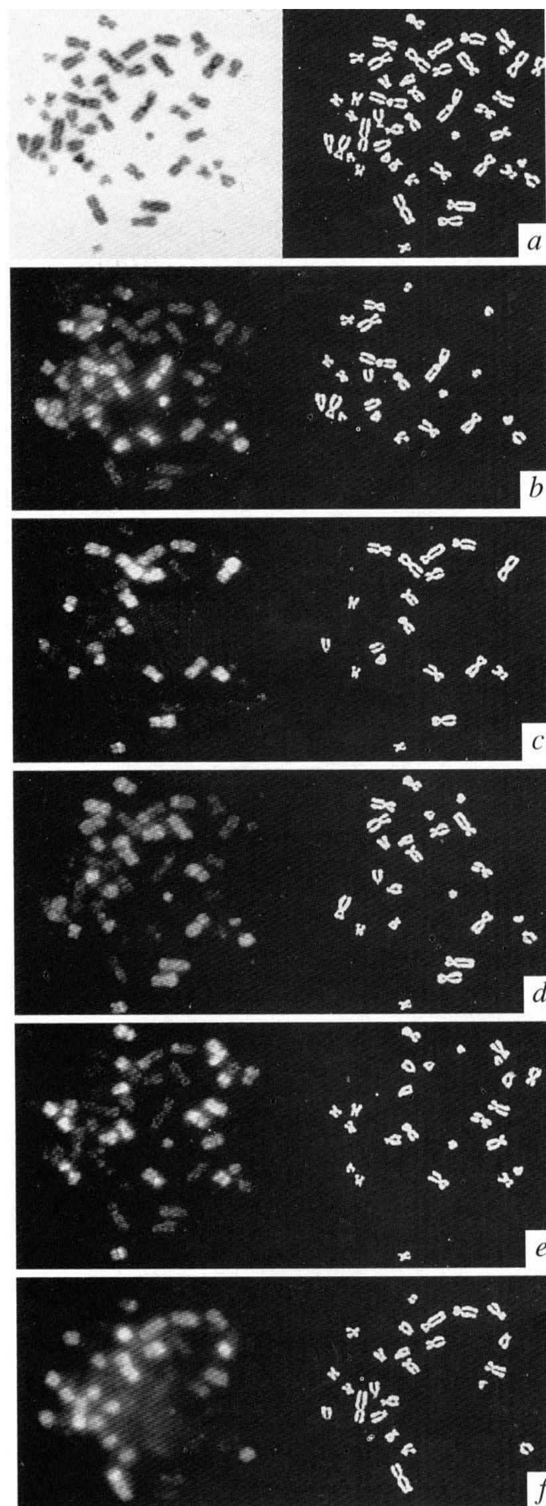

Fig. 1 Normal male metaphase spread after hybridization with a 27 DNA-probe cocktail. The left column of the figure shows the unprocessed fluorescence source images and the right column the segmentation masks computed for each fluor. *a*, DAPI: the DAPI source image was inverted to produce a G-band like pattern. *b*, FITC. *c*, Cy3. *d*, Cy3.5. *e*, Cy5. *f*, Cy7.

posite gray value image, where the material from each chromosome is encoded with a gray value; and 7) final presentation of the hybridization results using a look-up table (LUT) that assigns a pseudocolour to each such gray value.

Colour karyotyping of human chromosomes

To test the feasibility of karyotyping chromosomes by multiplex-FISH (M-FISH), chromosome painting probes representing the 22 autosomes and two sex chromosomes were combinatorially labelled, mixed, and cohybridized to normal metaphase spreads prepared from peripheral blood lymphocytes. In the case of chromosomes 3, 5 and 11 separate p and q arm probes were used; thus a total of 27 different fluor combinations were tested in this experiment (Table 2). The DNA probes were generated by microdissection and subsequent PCR amplification and labelled by nick translation (see Methods). As expected, probes labelled with equal amounts of different fluors did not give equal signal intensities for each fluor, reflecting the fact that the filter sets were selected to maximize spectral resolution rather than throughput. To diminish signal intensity differentials, probe concentrations for the hybridization mix had to be established carefully in a large number of control experiments. (For the optimized hybridization conditions, see Methods.)

A combinatorial labelling scheme was established to simutaneously label all of the 22 autosomes and the sex chromosomes (Table 2). After hybridization with the 27 probe cocktail, the fluorescence source images for DAPI, FITC, Cy3, Cy3.5, Cy5 and Cy7 were collected and the segmentation masks computed for each fluor (Fig. 1). After subtracting the interchromosomal fluorescence background from the chromosomal fluorescence, the mean of the intrachromosomal fluorescence intensities was used to calculate the threshold for creating the individual segmentation mask of each fluor (Fig. 2). Discrimination of hybridization positive and hybridization negative chromosomes was not problematic and was accomplished within a second or two.

The combinatorial labelling scheme generated a spectral signature for each chromosome (Table 2). Each of the fluor segmentation masks was interrogated by the computer program in order to establish the fluor composition of all chromosomes. For example, chromosome 7 material is labelled with FITC, Cy3 and Cy3.5 while chromosome 9 material is labelled with FITC, Cy3 and Cy5. Each of the individual chromosomes (or chromosomal segments) identified in the fluor masks on the basis of their fluor composition (its boolean spectral signature) is displayed on the computer monitor next to the DAPI image of the metaphase spread. This provides a simple means for the operator to confirm chromosome identity and to assess any chromosomal abnormalities seen in the final gray value or pseudocoloured composite images.

The karyotype of a normal male was examined using this technique. Multiple chromosome spreads (20) were analysed; the spectral signature for each chromosome was computed (Fig. 3*a*). The p and q arms of chromosome 3, 5 and 11 were labelled differentially, reflecting the use of the arm specific probes. A normal karyotype was attained as expected (Fig. 3*b*). The high efficiency of the chromosome painting achieved and the reproducibility of these results through many additional experiments using either 24

© 1996 Nature Publishing Group http://www.nature.com/naturegenetics

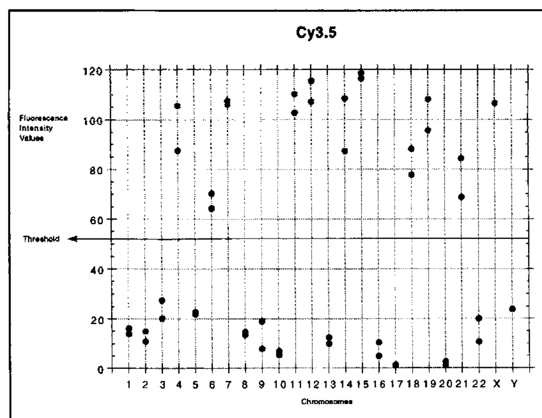

Fig. 2 Plot of the relative fluorescence intensity values for the fluor Cy3.5. Each pair of dots represents the mean fluorescence intensity values of the respective chromosome homologues. All chromosomes showing fluroescence above the threshold are represented in the Cy 3.5 mask. Note that chromosomes that were labelled with fluors very close to Cy3.5 in the spectrum, Cy3 or Cy5, but not with Cy3.5 do not have significantly increased intensity values. For example, chromosome 16 was labelled with Cy3 and Cy5 only. The intensity values over these chromosomes were no higher than any other chromosome not labelled with Cy3.5. This demonstrates the specificity of the filter used, thus allowing the unequivocal discrimination of hybridization positive and negative chromosomes.

or 27 colour labelling protocols indicated that karyotype analysis by M-FISH was feasible.

M-FISH analysis of chromosomal abnormalities

To further test the utility of M-FISH for karyotype analysis, we examined a set of five patient samples in which various chromosomal alterations had been detected by conventional cytogenetic banding techniques. These samples were provided by the Yale University Clinical Cytogenetics Laboratory and were analysed in a blind fashion using a pool of 24 whole chromosome painting probes. The karyotypes were determined by M-FISH for each of the five patient specimens (Fig. 4). The chromosomal abnormalities observed were as follows: BM2486, a chromosome 5;8 translocation (Fig. 4*a*); 10608, a 2;14 translocation (Fig. 4*b*); BM2645, a 2;3 translocation plus a deletion in 6q (Fig. 4*c*); BM2527, a 15;17 translocation (Fig. 4*d*), and BM3149, a 3;5 and a 6;12 translocation, loss of one copy of chromosomes 5 and 12 and trisomy of chromosome 8 (Fig. 4*e*). In each case, the chromosomal alterations seen by M-FISH were identical to those identified by cytogenetic banding. Furthermore, using the G-banding pattern generated by the DAPI fluorescence and measuring the fractional chromosome length of the translocation chromosomes, it was possible to infer the cytogenetic band implicated in the translocation breakpoints using fractional chromosome length/cytogenetic band conversion charts[17,18], which again agreed with the cytogenetic data. It should be noted, however, that differential chromosome condensation could lead to discordance between fractional length measurements and cytogenetic band assignments.

In some instances where the chromosomes are highly condensed there is an additional colour generated at the site of the translocation breakpoints (Fig. 4*a*, *c*, *d*). This is due to the blending of colours by fluorescence flaring at the junctions of the individual chromosome painting probe domains. This colour blending is not observed when more extended, prometaphase chromosomes, are examined (Fig. 4*b*), or when the fluor composition of both translocation chromosomes have only a single fluor difference (for example, Fig. 4*d*), where chromosome 15 was labelled with FITC, Cy3.5 and Cy7, and chromosome 17 was labelled with FITC and Cy7 only. However,

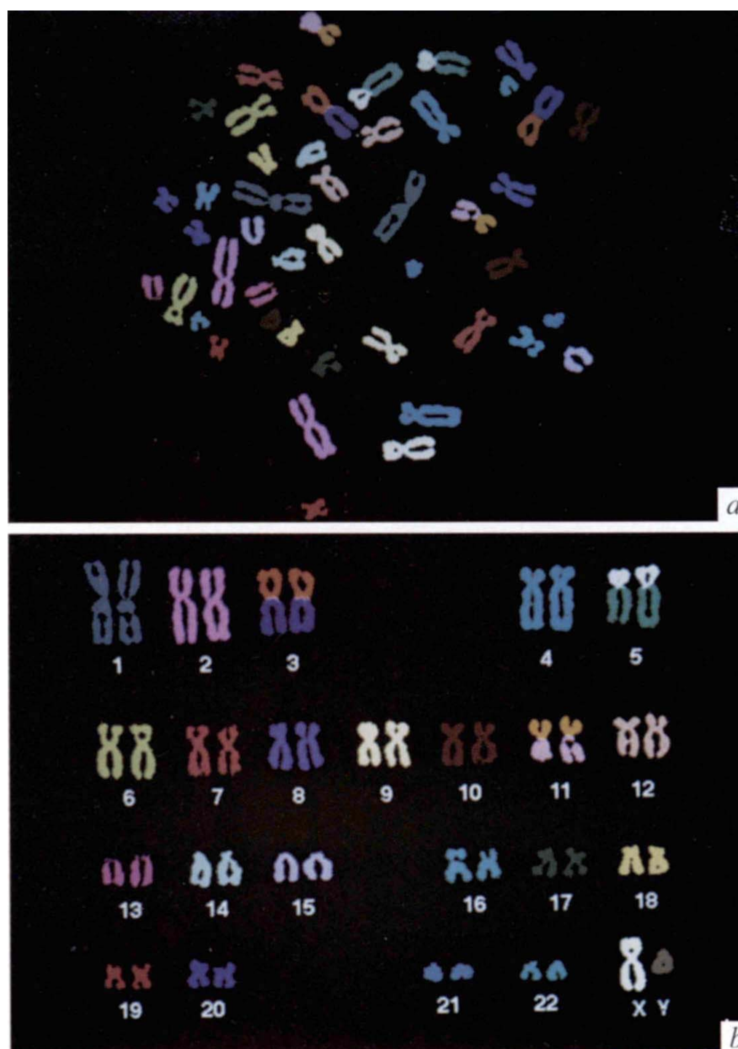

Fig. 3 *a*, Metaphase spread of Fig. 1 as a pseudocoloured image. For chromosomes 3, 5 and 11, the p and q arms were labelled differently. *b*, Final karyotype generated on the basis of the boolean spectral signature.

© 1996 Nature Publishing Group http://www.nature.com/naturegenetics

Fig. 4 Examples of chromosomal abnormalities detected by 24 colour M-FISH. *a*, BM2486: 46, XX, t(5;8). *b*, 10608: 46, XY, t(2;14). *c*, BM2645: 46, XX, t(2;3), del (6q) (arrow). *d*, BM2527: 46, XY, t(15;17). *e*, BM3149: 45, XY, −3, +der(5)t(3;5), der(6)t(6;12), +8, −12. Samples (*a*) and (*b*) were from individuals with constitutional translocations. Samples (*c*), (*d*) and (*e*) were from patients with acute myeloid leukaemia.

due to the fluorescence flaring of the Cy3.5 of chromosome 15 (Fig. 4*d*) the translocated chromosomal material from 17q appears smaller than would be expected for the recurring t(15;17)(q22;q11.2–12) in acute promyelocytic leukaemia (AML-M3). Colour blending also occurs at sites where two different chromosomes overlap in the spread. However, by examining several spreads potential problems in chromosome characterization can be avoided.

Previous studies have shown that G or R banding profiles can be generated by hybridization with oligonucleotides complementary to LINE or SINE (Alu) sequences[19,20]. Although such banding probes were not used in these experiments, it is clearly feasible to do so. However, since it is preferable to use a fluor that does not contribute to the spectral signature of the chromosomal DNAs to label the banding probe, we chose not to include hybridization banding in our protocol until we could refine the spectral resolution between Cy5 and Cy5.5. This would provide the additional fluor, Cy5.5, needed for such experiments.

We also examined several cell lines derived from patients with squamous cell carcinoma of the head and neck. These cell lines possess extensive chromosomal rearrangements and many of their chromosomes were designated only as 'marker' chromosomes when analysed by a board certified cytotechnologist using conventional cytogenetic banding procedures. One of these cell lines, HTB43, contains multiple clones with chromosome number varying between 48 and 62; many of these chromosomes are so extensively rearranged that conventional karyotype analysis is extremely difficult. Thus, finding cells that would give equivalent karyotypes by both cytogenetic banding and M-FISH is extremely difficult. In spite of this caveat, we demonstrated that M-FISH can identify complex chromosomal rearrangements not read-

© 1996 Nature Publishing Group http://www.nature.com/naturegenetics

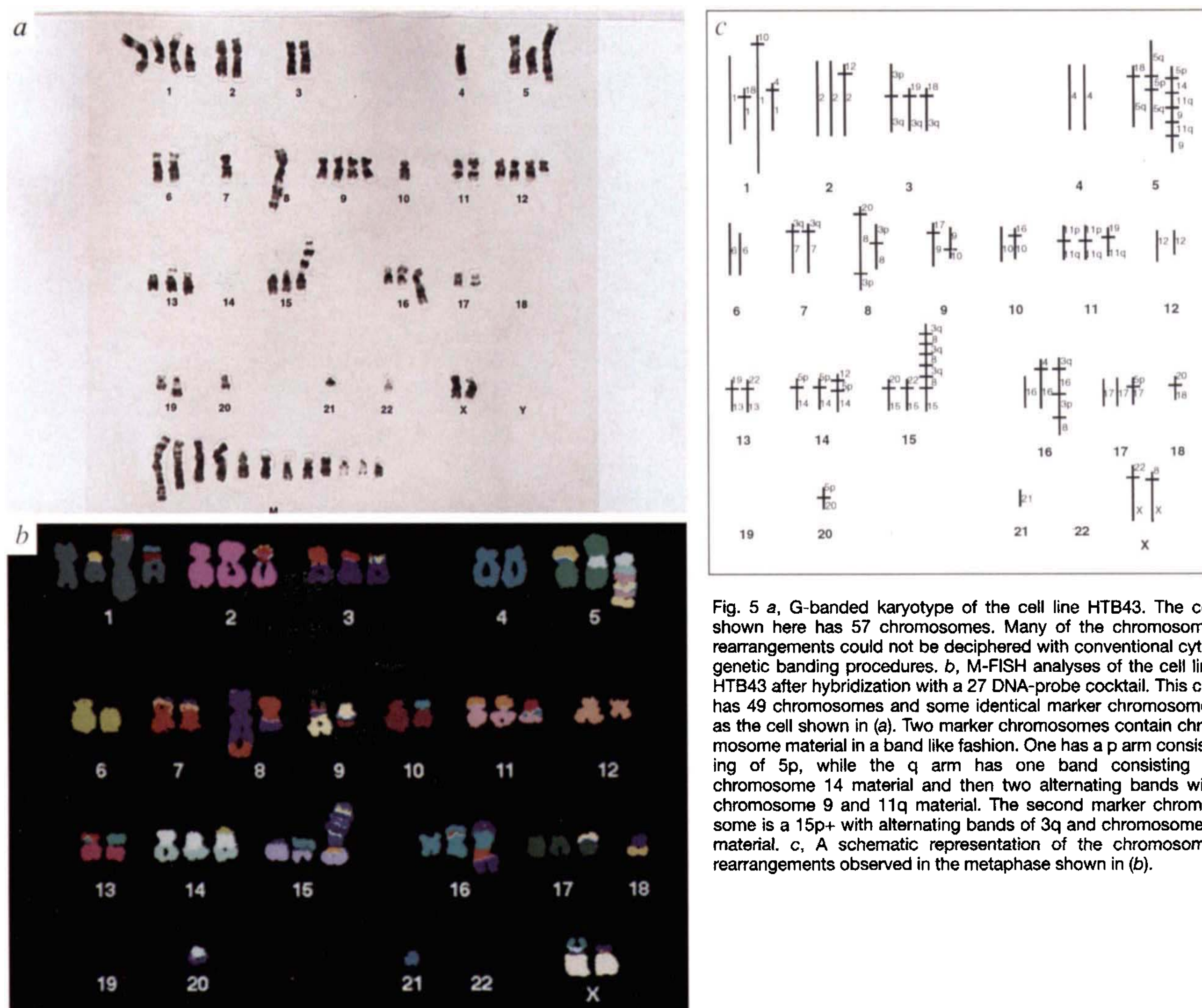

Fig. 5 *a*, G-banded karyotype of the cell line HTB43. The cell shown here has 57 chromosomes. Many of the chromosomal rearrangements could not be deciphered with conventional cytogenetic banding procedures. *b*, M-FISH analyses of the cell line HTB43 after hybridization with a 27 DNA-probe cocktail. This cell has 49 chromosomes and some identical marker chromosomes as the cell shown in (*a*). Two marker chromosomes contain chromosome material in a band like fashion. One has a p arm consisting of 5p, while the q arm has one band consisting of chromosome 14 material and then two alternating bands with chromosome 9 and 11q material. The second marker chromosome is a 15p+ with alternating bands of 3q and chromosome 8 material. *c*, A schematic representation of the chromosomal rearrangements observed in the metaphase shown in (*b*).

ily identified by conventional cytogenetic techniques (Fig. 5). M-FISH karyotype of an HTB43 cell with 49 chromosomes identified the chromosomal partners in numerous chromosomal translocations; some of the chromosomal rearrangements are extremely complex (Fig. 5*b*, *c*).

M-FISH with band probes and YAC clones

To determine if M-FISH could be done efficiently using regionally localized and genetically less complex probe mixtures, a set of three band specific probes from chromosome 6 were used with 19 non-chimaeric YAC clones and two whole chromosome painting probes (for chromosomes X and 8). We demonstrated that both band specific clone pools and individual YAC clones with inserts as small as 0.5 kb can be imaged and their spectral signatures correctly identified by our computer algorithms (data not shown). While the ultimate sensitivity of M-FISH has yet to be established, it should be possible to assemble probe panels to address a broad spectrum of specific biological and clinical questions.

Discussion

We demonstrate here the feasibility of discriminating up to 27 DNA probes hybridized simultaneously to metaphase chromosomes and document the ability to do karyotype analysis by multicolour FISH. Simple and complex translocations, interstitial deletions and insertions, chromosomal aneusomies and double minute chromosomes could be identified on a global basis quite rapidly. Furthermore, we were able to identify numerous chromosomal rearrangements that could not be classified by standard cytogenetic studies. Typically 5–10 metaphase spreads per case were analysed.

Defining the optimum fluor composition for each of the chromosome painting probes was done empirically through multiple hybridization experiments. Once the probe composition parameters were established, there was very little variation from one batch of probe to the next, thus highly reproducible results were obtained. These observations suggest that karyotyping by hybridization could be established in most laboratories skilled in FISH-techniques. The current software takes about 10 minutes to analyse one metaphase spread, however further refinements should reduce the analytical time significantly. Importantly, the M-FISH method is also amenable to full automation.

While we were readily able to visualize band specific probes derived from chromosome 6 and individual YAC clones containing DNA inserts of 0.5–1.0 Mb, we

© 1996 Nature Publishing Group http://www.nature.com/naturegenetics

have not yet tested systematically, the resolution limits of the chromosome painting probes to detect abnormalities that are routinely problematic for cytogeneticists, such as small telomeric translocations, interstitial deletions or duplications. Such studies are in progress. Since whole chromosome painting probes used in the absence of chromosome banding will not detect certain chromosomal abnormalities, for example, pericentric inversions, it is important to combine painting and banding techniques to maximize the cytogenetic information obtainable. Although pericentric inversions could be revealed if appropriate arm specific probes were used, direct banding would be preferable. Increasing the spectral resolution between Cy5 and Cy5.5, a goal that should be obtainable in the near future, would provide the additional fluor needed for banding by hybridization. The utility of M-FISH also could be extended by designing specific probe sets that stain particular chromosomal regions; telomere proximal probes for the detection of cryptic translocations, or band probes spanning translocation breakpoints associated with discrete subsets of leukemias or lymphomas. Recent advances in constructing physical and genetic maps of the human genome[21,22] and chromosome microdissection[23–27] should greatly facilitate the selection of clones to construct probe sets for specific diagnostic applications. While additional experimental evaluation will be required to define the practical advantages and technical limitations of M-FISH fully, it appears to be a versatile technique that should complement existing cytogenetic methods, particularly in characterizing complex karyotypes.

The application of M-FISH to interphase cytogenetics has yet to be explored in detail. While preliminary experiments have demonstrated the feasibility of delineating the signals from 20 chromosome-specific alphoid DNA repeat sequences by optical sectioning of cultured cells (data not shown), additional experiments will be required to determine if M-FISH can be used effectively to enumerate chromosomes in intact cells or tissues. However, with appropriate 3-D laser scanning imaging systems or algorithms for deconvoluting optical section images recorded by CCD cameras, it should be possible to analyse the intranuclear organization of whole chromosomes, defined chromosomal domains or multigene families as a function of developmental status, cell cycle stage or disease state. Such studies could provide important new insights into the architectural organization and dynamics of chromosome structure as a function of nuclear metabolic activity.

The potential utility of M-FISH is not limited to cytogenetic areas. For example, chromosomal painting probes from human or other species could be used to readily determine the location and extent of synteny between different species, thus enhancing our understanding of chromosome evolution. M-FISH could also be used to assess the presence or absence of infectious agents, define microbial serotypes or simultaneously determine not only the identity of an infectious agent but also its susceptibility or resistance to antibiotics or drugs. Multiplex gene mapping and the ability to quantitatively assess the levels of multiple mRNAs or proteins in a single cell or to determine if they exhibit different intracellular distributions could be applied to a myriad of interesting biological questions. Multiparametric imaging not only increases information throughput, it makes more efficient use of biological materials and can reveal spatial and temporal correlations, as well as mosaicisms, that otherwise might be difficult to establish reliably.

It has been suggested that the next generation of cytogenetic techniques would be molecularly based and employ multiple hybridization probes, each discriminated by a unique colour code[3,4,7,10,28]. Our studies provide a first step toward achieving this objective.

Methods

Software for analysis of combinatorially labelled probes. The program was developed using an image analysis package (BDS-Image/Oncor-Image) implemented on a Macintosh Quadra 900. Image shifts caused by optical and mechanical imperfections were corrected by alignment of the center of mass of one chromosome in each image according to a procedure described[29] and modified[30]. The DAPI image was used to define the morphological boundary of each chromosome. Chromosome segmentation was assisted by pre-filtering of each image through a top-hat filter[30,31], with the mode of the gray level histogram of the filtered DAPI image used as the threshold. For each fluor optical background was eliminated by subtracting the interchromosomal fluorescence intensity from the chromosomal fluorescence. The mean of the intrachromosomal fluorescence intensities was used to calculate the threshold for the individual segmentation mask of each fluor. Individual DNA targets were assigned distinct gray values depending on the boolean signature of each probe, that is, the combination of fluors used to label it. Finally, a look-up-table was used to assign each DNA target a pseudocolour depending on this gray value.

DNA probes. All microdissected probes were provided by J. Trent, P. Meltzer and M. Bittner (National Center for Human Genome Research, Bethesda, MD). These probes give a very uniform labelling of the target region. The detailed protocols for microdissection and PCR amplification are described[23–27]. For some chromosomes different DNA-probes for the p- and the q-arms were available, namely 2, 3, 4, 5, 10, 11, 16, 18, and Y. For all other chromosomes microdissected probes painting the entire chromosome were used.

Probe labelling. Fluorescein, Cy3, and Cy5 were linked to dUTP for direct labelling. Cy3.5 and Cy7 were available as avidin or anti-digoxigenin conjugates for secondary detection of biotin- or digoxigenin-labelled probes. They were synthesized using conventional N-succinamide ester coupling chemistry. For each probe one to four separate nick translation reactions were necessary, each with a single fluor-, biotin- or digoxigenin-labelled triphosphate (Table 2).

FISH. The reporters and the exact probe concentrations used for each chromosome paint were: 1-FITC: 110 ng; 2p-Dig: 60 ng; 2q-Dig: 200 ng; 3-Cy3: 150 ng; 3p-Cy5: 90 ng; 3q-Dig: 70 ng; 4p-Bio: 30 ng; 4q-Bio: 140 ng; 5p-Cy3: 80 ng; 5p-Dig: 70 ng; 5p-Bio: 80 ng; 5p-Cy5: 100 ng; 5q-Cy3: 170 ng; 5q-Dig: 110 ng; 6-FITC: 110 ng; 6-Bio: 60 ng; 7-FITC: 40 ng; 7-Cy3: 40 ng; 7-Bio: 20 ng; 8-Cy5: 110 ng; 8-Dig: 60 ng; 9-FITC: 110 ng; 9-Cy3: 140 ng; 9-Cy5: 120 ng; 10p-Cy5: 90 ng; 10q-Cy5: 120 ng; 11p-Bio: 20 ng; 11p-Cy5: 50 ng; 11p-FITC: 20 ng; 11p-Dig: 20 ng; 11q-Bio: 20 ng; 11q-Cy5: 60 ng; 12-Cy3: 70 ng; 12-Bio: 20 ng; 12-Dig: 60 ng; 13-FITC: 20 ng; 13-Cy3: 40 ng; 13-Dig: 40 ng; 14-Bio: 20 ng; 14-Cy5: 50 ng; 14-Dig: 40 ng; 15-FITC: 20 ng; 15-Bio: 20 ng; 15-Dig: 40 ng; 16p-Cy3: 70 ng; 16p-Cy5: 70 ng; 16q-Cy3: 90 ng; 16q-Cy5: 70 ng; 17-FITC: 30 ng; 17-Dig: 60 ng; 18p-Bio: 20 ng; 18p-Dig: 60 ng; 18q-Bio: 40 ng; 18q-Dig: 80 ng; 19-Cy3: 30 ng; 19-Bio: 20 ng; 19-Cy5: 60 ng; 20-FITC: 20 ng; 20-Cy5: 60 ng; 20-

© 1996 Nature Publishing Group http://www.nature.com/naturegenetics

Dig: 20 ng; 21q-FITC: 70 ng; 21q-Bio: 70 ng; 21q-Cy5: 90 ng; 22q-FITC: 100 ng; 22q-Cy5: 120 ng; X-Cy3: 40 ng; X-Bio: 20 ng; Yp-FITC: 10 ng; Yp-Cy3: 10 ng; Yq-FITC: 5 ng; Yq-Cy3: 5 ng. Thus, the total mass of labelled probe was 4.45 μg. Cot1 DNA (70 μg) and 20 μg salmon DNA were added for an overnight ethanol precipitation. On the next day the mixture of probes was resuspended in 10 μl of a conventional hybridization cocktail with 50% formamide, 2× SSC and 2% dextran sulfate.

Probes were denatured and hybridized for two to three nights at 37 °C to metaphase chromosome spreads. The slides were washed at 45 °C in 50% formamide/2× SSC three times followed by three washes at 60 °C in 0.1× SSC to remove excess probe. After a blocking step in 4× SSC/3% bovine serum albumin for 30 min at 37 °C the biotinylated probes were detected with avidin-Cy3.5 and the dig-labelled probes with anti-dig-Cy7. Fluorescein-dUTP, Cy3-dUTP and Cy5-dUTP did not require any immunological detection step. After final washes at 45 °C with 4× SSC/0.1% Tween-20 three times, mounting medium and a coverslip were applied, and the hybridization signals from each fluor were imaged using the filters sets listed in Table 1.

Cell lines. The cell lines derived from patients with squamous cell carcinoma were obtained from the American Type Culture Collection (ATCC).

Acknowledgements

We thank T. Yang-Feng and L. Gibson for karyotyped clinical samples for M-FISH analyses; T. Haaf for input during the initial stages of this work; and N. Mokady for propagation of YAC clones and cell lines. This work was supported by grant 93-018 from the Department of the Army. M.R.S. was a recipient of a grant from the Deutsche Forschungsgemeinschaft (Sp 460/1-1).

Received 11 January; accepted 20 February 1996.

1. Gray, J.W. *et al.* Molecular cytogenetics: diagnosis and prognostic assessment. *Curr. Opin. Biotech.* **3**, 623–631 (1992).
2. Xing, Y. & Lawrence, J.B. Molecular cytogenetics: applications of FISH to chromosomal aberrations in cancer genetics. In *The causes and consequences of chromosomal aberrations*. (ed. Kirsch, I.R.) 3–28 (CRC Press, Boca Raton, 1993).
3. Nederlof, P.M. *et al.* Three color fluorescence *in situ* hybridization for the simultaneous detection of multiple nucleic acid sequences. *Cytometry* **10**, 20–27 (1989).
4. Nederlof, P.M. *et al.* Multiple fluorescence *in situ* hybridization. *Cytometry* **11**, 126–131 (1990).
5. Ried, T., Baldini, A., Rand, T.C. & Ward, D.C. Simultaneous visualization of seven different DNA probes by *in situ* hybridization using combinatorial fluorescence and digital imaging microscopy. *Proc. Natl. Acad. Sci. USA* **89**, 1388–1392 (1992).
6. Dauwerse, J.G., Wiegant, J., Raap, A.K., Breuning, M.H. & van Ommen, G.J.B. Multiple colors by fluorescence *in situ* hybridization using ratio-labelled DNA probes create a molecular karyotype. *Hum. Mol. Genet.* **1**, 593–598 (1992).
7. Nederlof, P.M., van der Flier, S., Vrolijk, J., Tanke, H.J. & Raap, A.K. Fluorescence ratio measurements of double-labeled probes for multiple *in situ* hybridization by digital imaging microscopy. *Cytometry* **13**, 839–845 (1992).
8. du Manoir, S.*et al.* Detection of complete and partial chromosome gains and losses by comparative genomic *in situ* hybridization. *Hum. Genet.* **90**, 590–610 (1993).
9. Ried, T., Landes, G., Dackowski, W., Klinger, K. & Ward, D.C. Multicolor fluorescence *in situ* hybridization for the simultaneous detection of probe sets for chromosomes 13, 18, 21, X and Y in uncultured amniotic fluid cells. *Hum. Mol. Genet.* **1**, 307–313 (1992).
10. Lengauer, C. *et al.* Chromosomal bar codes constructed by fluorescence *in situ* hybridization with Alu-PCR products of multiple YAC clones. *Hum. Mol. Genet.* **2**, 505–512 (1993).
11. Popp, S. *et al.* A strategy for the characterization of minute chromosome rearrangements using multiple color fluorescence in situ hybridization with chromosome specific DNA libraries and YAC clones. *Hum. Genet.* **92**, 527–532 (1993).
12. Wiegant, J. *et al.* Multiple and sensitive fluorescence *in situ* hybridization with rhodamine-, fluorescein-, and coumarin-labeled DNAs. *Cytogenet. Cell Genet.* **63**, 73–76 (1993).
13. Ernst, L.A., Gupta, R.K., Mujumdar, R.B. & Waggoner, A.S. Cyanine dye labeling reagents for sulfhydryl groups. *Cytometry* **10**, 3–10 (1989).
14. Mujumdar, R.B., Ernst, L.A., Mujumbar, S.R. & Waggoner, A.S. Cyanine dye labeling reagents containing isothiocyanate groups. *Cytometry* **10**, 11–19 (1989).
15. Yu, H. *et al.* Cyanine dye dUTP analogs for enzymatic labeling of DNA probes. *Nucl. Acids Res.* **22**, 3226–3232 (1994).
16. Waggoner, A. Covalent labeling of proteins and nucleic acids with fluorophores. *Meth. Enzym.* **246**, 362–373 (1995).
17. Francke, U. Digitized and differentially shaded human chromosome ideograms for genomic applications. *Cytogenet. Cell Genet.* **65**, 205–219 (1994).
18. Bray-Ward, P. *et al.* Integration of the cytogenetic, genetic and physical maps of the human genome by FISH mapping of CEPH YAC clones. *Genomics* (in the press).
19. Baldini, A. & Ward, D.C. *In situ* hybridzation banding of human chromosomes with Alu-PCR products: a simultaneous karyotype for gene mapping studies. *Genomics* **9**, 770–774 (1991).
20. Matera, A.G. & Ward, D.C. Oligonucleotide probes for the analysis of specific repetitive DNA sequences by fluorescence *in situ* hybridization. *Hum. Mol. Genet.* **1**, 535–539 (1992).
21. Chumakov, I.M. *et al.* A YAC contig of the human genome. *Nature* **377**, 175–299 (1995).
22. Hudson, T.J. *et al.* An STS-based map of the human genome. *Science* **270**, 1945–1954 (1995).
23. Meltzer, P.S., Guan, X.Y., Burgess, A. & Trent, J. Rapid generation of region specific probes by chromosome microdissection and their application. *Nature Genet.* **1**, 24–28 (1992).
24. Guan, X.Y., Trent, J.M. & Meltzer, P.S. Generation of band-specific painting probes from a single microdissected chromosome. *Hum. Mol. Genet.* **2**, 1117–1121 (1993).
25. Guan, X.Y., Meltzer, P.S. & Trent, J.M. Rapid generation of whole chromosome painting probes (WCPs) by chromosome microdissection. *Genomics* **22**, 101–107 (1994).
26. Guan, X.Y., Meltzer, P.S., Burgess, A. & Trent, J.M. Complete coverage of chromosome 6 by chromosome microdissection: Generation of 14 band region-specific probes. *Hum. Genet.* **95**, 637–640 (1995).
27. Guan, X.Y. *et al.* Chromosome arm painting probes. *Nature Genet.* **12**, 10–11 (1996).
28. Ledbetter, D.H. The 'colorizing' of cytogenetics: is it ready for prime time? *Hum. Mol. Genet.* **5**, 297–299 (1992).
29. Waggoner, A. *et al.* Multiple spectral parameter imaging. *Meth. Cell. Biol.* **30**, 449–478 (1989).
30. du Manoir, S. *et al.* Quantitative analysis of comparative genomic hybridization. *Cytometry* **9**, 21–49 (1995).
31. Smith, T.G. Jr., Marks, W.B., Lange, G.D., Sheriff, W.H. Jr. & Neale, E.A. Edge detection in images using Marr-Hildreth filtering techniques. *J. Neurosci. Meth.* **26**, 75–81 (1988).