

# Classifying by colors: FISH-based genome analysis

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**Abstract.** In recent years a fascinating evolution of different multicolor fluorescence in situ hybridization (FISH) technologies could be witnessed. The various approaches to cohybridize multiple DNA probes in different colors opened new avenues for FISH-based automated karyotyping or the simultaneous analysis of multiple defined regions within the genome. These developments had a remarkable impact on microscopy design and the usage of highly sensitive area imagers. In addition, they

led to the introduction of new fluorochromes with appropriate filter combinations, refinements of hybridization protocols, novel probe sets, and innovative software for automated chromosome analysis. This paper attempts to summarize the various multicolor approaches and discusses the application of the individual technologies.

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At the time of their discovery in the 19<sup>th</sup> century chromosomes were already linked to colors. Because of their affinity for certain stains Waldeyer (1888) dubbed the threadlike structures “chromosomes” which is derived from the Greek words chroma (= color) and soma (= body), and means “colored body”. However, chromosomes had to travel through various color ages, starting from the “dark ages” (Hsu, 1979) to arrive finally at the “kaleidoscope-age” (Speicher and Ward, 1996) where multicolor-FISH technologies made the “colored bodies” an especially apt derivation. This is illustrated in Fig. 1, which depicts side by side the first and present-day images of human chromosomes and karyotypes.

In recent years, FISH became an important addition to traditional karyotyping, which based on the work by Zech and Caspersson (Caspersson et al., 1968, 1970) depends on the analysis of characteristic banding patterns along the length of each chromosome. However, chromosome karyotyping by conventional cytogenetic banding methods is time consuming, expensive, and has a limited resolution. Particularly proble-

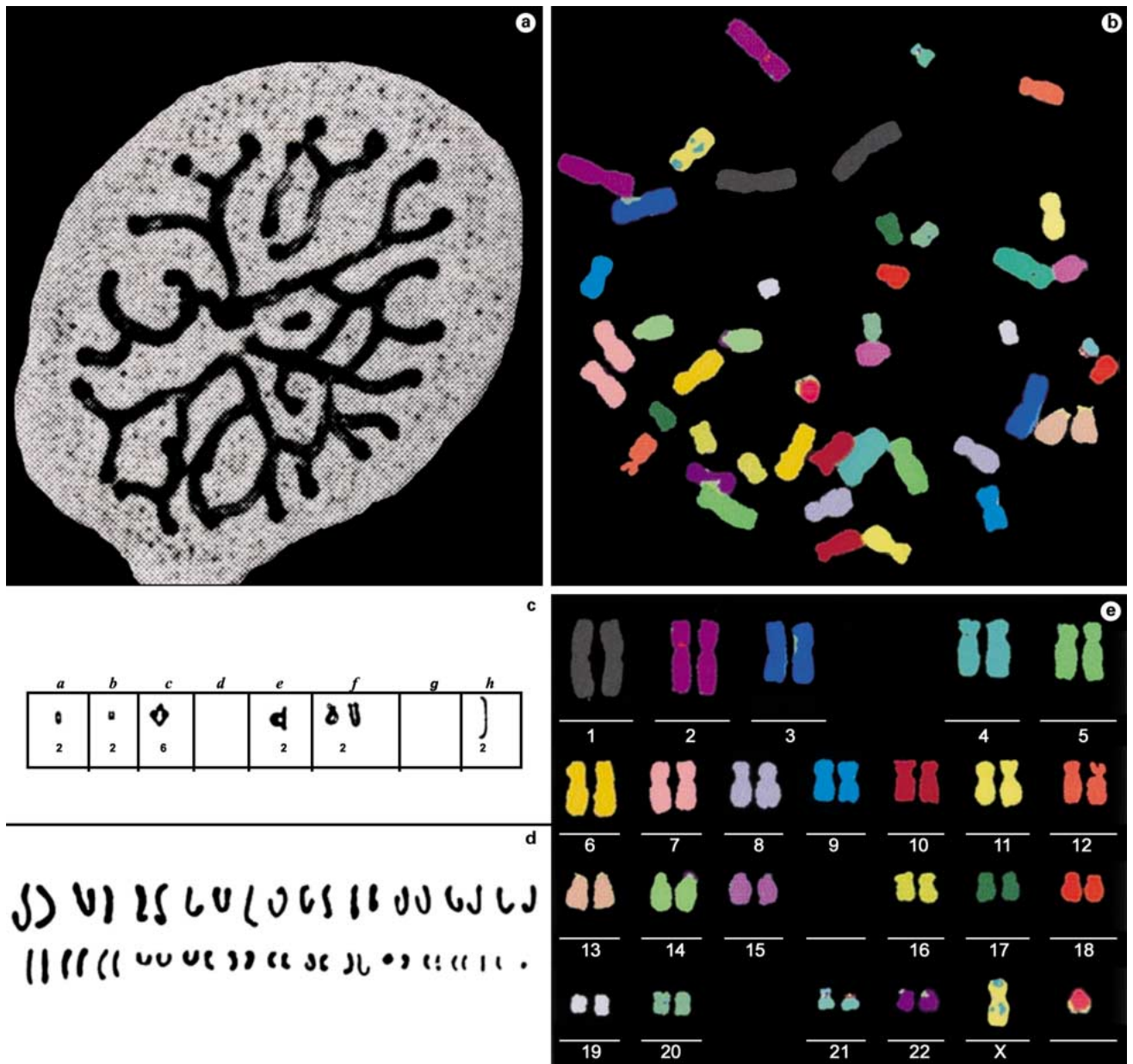
matic are the analysis of extensively rearranged chromosomes in tumor cytogenetics and the identification of marker chromosomes. Thus, chromosome-specific DNA painting probes or other region-specific probes (e.g. YACs, BACs, PACs, cosmids, etc.) were applied for the unequivocal identification of certain chromosomes or selected DNA regions within metaphase spreads. Applying highly sensitive area imagers such as cooled charge coupled device (CCD) cameras or interferometers and appropriate filter sets several fluorochromes can spectrally be resolved and the intensity of hybridization signals can accurately be quantified. Thus, FISH is ideally suited for the simultaneous detection of multiple hybridization probes.

The discrimination of many more targets than the number of spectrally resolvable fluorochromes can be achieved using either combinatorial (Nederlof et al., 1989, 1990; Ried et al., 1992a, b; Lengauer et al., 1993; Popp et al., 1993; Wiegant et al., 1993; Speicher et al., 1996a; Schröck et al., 1996) or ratio labeling (Dauwerse et al., 1992; Nederlof et al., 1992; du Manoir et al., 1993; Morrison et al., 1997; Tanke et al., 1999) strategies (Fig. 2). All currently used multicolor FISH technologies use either technology or a combination of both. The combinatorial labeling strategy provides the simplest way to label probes in a multiplex fashion because each probe fluorochrome is either completely absent (0) or present (1) resulting in a “Boolean spectral signature” of each probe (Fig. 2a). Image analysis is thus more amenable to simple automation. Ratio coding uses hybridization probes containing different proportions of two or more distinguishable labels to stain each target. Discrimination is achieved by highly accurate fluorescence intensity measure-

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**Fig. 1 . (a)** One of the first images of human chromosomes made by the German pathologist J. Arnold in 1879. Arnold examined carcinoma and sarcoma cells because their voluminous nucleus facilitated analysis. The drawing shows a human sarcoma cell (Arnold, 1879). **(b)** Staining of all 46 chromosomes of a human cell simultaneously in different colors by M-FISH. **(c)** The first karyogram of human chromosomes made by J. Moore and G. Arnold in 1906. Chromosomes were observed at meiotic division I. Because the paired homologous chromosomes are connected by the synaptonemal complex they were drawn as characteristic double structures. As 16 double

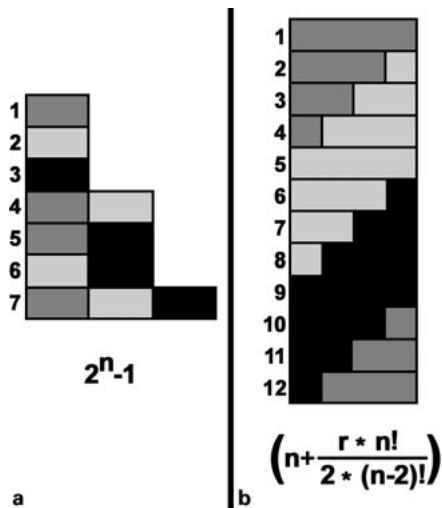
structures were counted the authors concluded that human cells have 32 chromosomes (Moore and Arnold, 1906). **(d)** A karyogram made by T. Painter in 1923, who used drawings from spermatogonia to sort chromosomes according to their size and shape. Chromosome number was estimated to be at 47 (Painter, 1923). **(e)** Multicolor classified karyogram of the normal male metaphase spread shown in (b), generated by an adaptive spectral classification approach for seven fluorochromes. The automated analysis is assisted by computer-generated false colors, which allow the generation of a karyotype within seconds.

ments, which determine not only the presence of a fluorochrome but also the ratios between the fluorochromes used for probe labeling. Therefore, ratio labeling has the potential to identify more DNA targets simultaneously using fewer fluorescent labels than the combinatorial labeling technique (Fig. 2b).

This paper will review the recent developments of various multicolor-FISH technologies, their applications, limitations and pitfalls, and further amendments.

### Multicolor karyotyping technologies using painting probes

For multicolor karyotyping with painting probes several approaches were developed, including multiplex FISH (M-FISH; Speicher et al., 1996a), spectral karyotyping (SKY; Schröck et al., 1996), color changing karyotyping (Henegariu et al., 1999), and combined binary ratio labeling (COBRA; Tanke



**Fig. 2.** Schemes illustrating the principles of the combinatorial and the ratio labeling technologies. **(a)** Combinatorial labeling employs the calculation of a “Boolean spectral signature” for each probe. Each probe is either completely absent (0) or present (1) depending on the intensity of the fluorochrome. Thus, when probes are labeled with different fluorochromes the ratio between these fluorochromes does not matter. The number of useful Boolean combinations for  $n$  fluorochromes is  $2^n - 1$ . In the example shown there are three fluorochromes (red, yellow, blue), yielding  $2^3 - 1 = 7$  different color combinations. At least five fluorochromes ( $2^5 - 1 = 31$ ) are needed for the generation of 24 different colors. **(b)** With ratio labeling different probes can be labeled with the same fluorochrome combinations (as shown for probes 2–4, 6–8, and 10–12). Different probes labeled with the same fluorochrome combination are distinguished based on the different proportions or ratios of the fluorochromes used. The number of combinations for  $n$  fluorochromes is given by the formula:

$$\text{Number of colors} = \left( n + \frac{r * n!}{2 * (n-2)!} \right)$$

where  $r$  is the number of ratios, which can be resolved for ratio labeling. In the example shown there are three fluorochromes and three ratios (used to label probes 2–4, 6–8, or 10–12, respectively). Thus the number of combinations is:

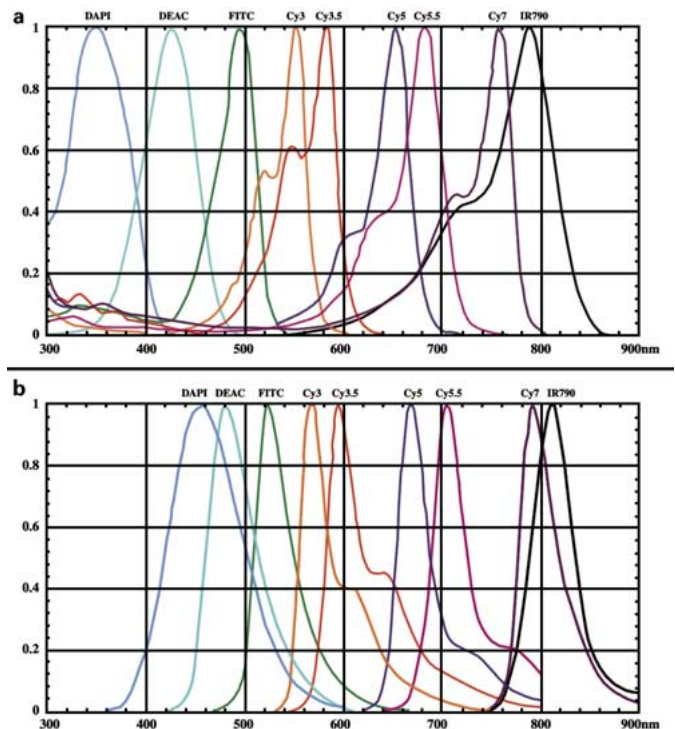
$$\left( 3 + \frac{3 * 3!}{2 * (3-2)!} \right) = \left( 3 + \frac{3 * 6}{2 * 1} \right) = \left( 3 + \frac{18}{2} \right) = 3 + 9 = 12$$

A further increase in the number of colors can be achieved by adding a binary label to some targets (see text).

et al., 1999). The first three methods use the combinatorial labeling strategy, COBRA employs both the combinatorial and the ratio labeling strategy. All technologies use similar, spectrally resolvable fluorochromes. The emission and excitation spectra of commonly used fluorochromes are illustrated in Fig. 3.

#### Multiplex-FISH (M-FISH)

M-FISH uses appropriate epifluorescence filter sets and computer software for the detection and evaluation with a standard epifluorescence microscope (Speicher et al., 1996a, 1996b, 2000; Eils et al., 1998; Azofeifa et al., 2000). Critical features are accurate alignment of source images, reduction of chromatic crosstalk, and quantitation of the intensity of each fluorochrome. M-FISH had a remarkable impact on microscopy design as most vendors are now offering epifluorescence microscopes with motorized eight-position filter wheels. The filter wheel reduces



**Fig. 3.** Normalized fluorescence excitation **(a)** and emission **(b)** spectra of 4'-6-diamidino-2-phenylindole (DAPI [a general DNA counterstain], absorption maximum: 350 nm; emission maximum: 456 nm), diethylamino-coumarin (DEAC, 426 nm; 480 nm), fluorescein (FITC, 490 nm; 520 nm), the cyanine dyes Cy3 (554 nm; 568 nm), Cy3.5 (581 nm; 588 nm), Cy5 (652 nm; 672 nm), Cy5.5 (682 nm; 703 nm), Cy7 (755 nm; 778 nm), and LaserPro IR 790 (785 nm; 801 nm). In many multicolor FISH applications the entire spectrum from the UV to the far infrared range is utilized. Despite a significant spectral overlap individual fluorochromes can be resolved without undesirable cross talk between detection channels using appropriate filter sets or an interferometer. Human vision is insensitive to light beyond ~ 650 nm, and therefore it is not possible to view the far-red fluorescence dyes by looking through the eyepiece of a conventional fluorescence microscope. Thus, fluorochromes Cy5, Cy5.5, Cy7, and LaserPro IR790 are only detectable by using a highly sensitive area imager, e.g. a cooled charge coupled device (CCD) camera.

mechanical noise to an extent that geometric image displacement is avoided. In addition, images from the different fluorochromes can be taken in a completely automated fashion.

A set of up to eight fluorochromes and corresponding optical filters spaced across the spectral interval 350–801 nm that give a high degree of discrimination between all possible fluorochrome pairs has so far been used for M-FISH (Fig. 3, Azofeifa et al., 2000). Epifluorescence filter cubes were constructed for spectral contrast between adjacent fluorochromes to attain the required selectivity (Eils et al., 1998). In order to reduce the crosstalk excitation and emission filters have to be narrow. However, exposure times are short as light yield in epifluorescence microscopes is extremely efficient resulting in a sufficient number of available photons. For example, typical exposure times are in the range of 0.5 s for DAPI, 3 s for DEAC, 3 s for FITC, 3 s for Cy3, 0.5 s for Cy3.5, 3 s for Cy5, 4 s for Cy5.5, and 4 s for Cy7 (Eils et al., 1998; Azofeifa et al., 2000). All images of a metaphase spread can thus be captured in less than 25 s.

### *Spectral karyotyping (SKY)*

Spectral karyotyping (SKY) employs an interferometer and Fourier analysis to obtain an image of the fluorescing sample. Interferometers operate by dividing the optical energy from a light source (e.g., via a beamsplitter, spectral cube, etc.) into two substantially equal beams of light. The two beams propagate in opposite directions. They are combined after one is permitted to pass through the sample and the interference pattern (i.e., the changes in intensity of the combined light) is detected. Such changes in intensity are related to the fluorescence of the sample. The interference pattern is detected with a CCD camera, so that both the interferometer and the camera capture an interferogram (Schröck et al., 1996).

Employing a triple-band pass filter all fluorochromes are excited and emitted at the same time. The properties of this filter determine the fraction of the spectrum, which is used to distinguish the multiple spectrally overlapping probes of each combinatorially labeled object pixel. The simultaneous excitation and emission of all fluorochromes, however, neglect the different fading properties of individual fluorochromes and that – depending on the characteristics of the objective used – fluorochromes may have different chromatic aberrations resulting in different focal planes. Exposure times are in the range of 1–2 min.

As commercial probe kits were readily available for the SKY system, it was soon, after its first description, used for numerous applications in pre- and postnatal diagnostics and tumor cytogenetics. These were recently reviewed intensively (Schröck and Padilla-Nash 2000).

### *Color changing karyotyping (CCK)*

Color changing karyotyping (CCK) (Henegariu et al., 1999) is based on combinatorial labeling, but uses only three fluorescent dyes for chromosome identification. For example, three fluorescent and three hapten-labeled nucleotides are used for probe labeling. After probe labeling and hybridization, grayscale images of the three directly labeled probes are captured. After recording of the coordinates of each metaphase the anti-fade mounting medium is rinsed away and the slide subjected to antibodies and avidin, which are labeled with the same three fluorochromes as the directly labeled probes. Images of the previously recorded metaphases are again captured in all three channels. To distinguish between the signals from the first and second recording CCK takes advantage of the difference in signal strength between directly and indirectly labeled probes. Subsequently the grayscale images are pseudocolored and merged with the first images. Analysis is primarily done by visual inspection. This approach represents the cheapest alternative of all published multicolor karyotyping systems. However, as metaphase spreads have to be captured twice and as image analysis is not automated it increases the workload and is time consuming.

### *Combined binary ratio labeling (COBRA)*

The potential of ratio labeling was impressively documented in 1992 by 12-color experiments in first attempts to create a “molecular karyotype” (Dauwerse et al., 1992). The COBRA technology is in some respect a continuation of this

work (Tanke et al., 1999). COBRA uses simultaneously the combinatorial and ratio labeling strategies. Three fluorochromes are used for ratio labeling of 12 chromosome painting probes (Fig. 2b). In order to label 24 chromosomes, a second set of 12 painting probes is labeled identically but is in addition tagged with a fourth fluorochrome. This additional tag represents a binary label, which similarly to a combinatorially labeled fluorochrome is either present or absent. The addition of further binary labels increases the multiplicity of this approach tremendously. For example, if three fluorochromes are used for the ratio labeling of 12 DNA probes one additional binary label (four fluorochromes altogether) would allow to distinguish 24 probes, two binary labels (five fluorochromes altogether) 48 probes, three binary labels (six fluorochromes altogether) 96 probes, and so on.

A weakness is that ratio labeling is more complicated in terms of probe labeling and more accurate fluorescence measurements are needed. In addition, it is more prone to hybridization variability artifacts.

## **Multicolor karyotyping technologies employing bar coding strategies**

The major drawback of using painting probes is that the sensitivity for the detection of intrachromosomal rearrangements is low. Deletions or duplications will be detected only if they result in significant size differences of the two homologous chromosomes, pericentric inversions will be detected only if they result in a considerable displacement of the centromere, which changes the shape of the chromosome. Paracentric inversions are not detectable by painting probes at all.

Thus, strategies employing the application of multiple sub-regional probes for the generation of FISH-based multicolor banding patterns were already thought for a long time to be an ideal tool for deciphering intrachromosomal rearrangements (Lichter et al., 1990; Ried et al., 1992; Lengauer et al., 1993). Recently, such “bar coding” strategies were extended to the entire genome to combine traditional banding capability with color classification (Müller et al., 1997, 1998).

### *Multicolor chromosome bar code for the entire human genome*

Alu-PCR products of various human/rodent somatic cell hybrids can be used to achieve a specific colored banding pattern for each chromosome in a single hybridization. These sets of subregional DNA probes are pooled into two probe sets, differently labeled and after hybridization detected by green and red fluorescence. This approach results in about 110 distinct signals per haploid chromosome set with a unique sequence of signals on each individual chromosome (Müller et al., 1997).

### *Cross-species color segmenting (Rx-FISH)*

This approach takes advantage of the fact that humans and gibbons have evolved from a common ancestor. The DNA has retained a high degree of homology but the gibbon chromosomes are extensively rearranged. As a result, probes generated by flow sorting from gibbon chromosomes hybridize to differ-

ent loci in the human genome. By labeling a probe set derived from two gibbon species (*Hylobates concolor* and *Hylobates syndactylus*) with three fluorophores seven different colors are generated, which segment individual human chromosomes in at least two and up to six segments and the entire human karyotype into 81 or 74 homologous colored segments. An attractive feature of this technique is that the segments have precise boundaries that are defined by evolutionarily derived translocations (Müller et al., 1998).

### Multicolor strategies with multiple subregional probes for defined locations

As resolution depends critically on the selection of probes, the analysis of the entire genome should not always be the first choice. Instead there are a number of diagnostic applications or defined biologic questions for which a specifically designed probe set should be superior as compared to the aforementioned methods. For example, several probe sets were developed for an effective analysis of subtelomere regions (National Institutes of Health and Institute of Molecular Medicine collaboration, 1996; Kingsley et al., 1997; Knight et al., 2000). Other strategies aimed at achieving a higher resolution for individual chromosomes (Chudoba et al., 1999). These developments will be summarized below.

#### Telomere integrity assays

There is substantial evidence that a number of unexplained cases of mental retardation may be caused by cryptic subtelomeric rearrangements (Flint et al., 1995; Knight et al., 1999). These rearrangements pose a diagnostic challenge as they tend to escape routine banding analysis. Some unbalanced translocations are detectable using either M-FISH (Uhrig et al., 1999), SKY (Schröck et al., 1997) or COBRA (Bezrookove et al., 2000). However, as telomere regions are often poorly represented in painting probes (Holinski-Feder et al., 2000) and as subtelomeric deletions will not be detectable with these approaches, alternative strategies employing subtelomeric probes were sought by several groups. All approaches try to screen all subtelomeric regions simultaneously in an efficient way with the best possible resolution.

In addition to a commercial kit, which subdivides a slide into 24 hybridization fields, one for each chromosome (Knight et al., 1997), multicolor approaches were developed. One multicolor FISH assay checks the telomere integrity of eight chromosomes simultaneously, thus three hybridizations are needed (Granzow et al., 2000). Telomere signals are evaluated by visual inspection. A new assay was called "M-TEL", which allows the simultaneous assessment of the telomere ends of 12 chromosomes (Brown et al., 2001) reducing the number of hybridizations to two. Analysis is done in an automated fashion by newly developed software, which allows the automated classification of multiple small region-specific probes (Saracoglu et al., 2001). The simultaneous analysis of all chromosome ends in a single multicolor hybridization was also reported recently (Henegariu et al., 2001).

**Table 1.** The resolution limits of any multicolor system do not depend entirely on the system itself but also on a variety of additional, system-independent factors. The table summarizes such parameters, which may have a substantial effect on resolution.

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Slide/chromosome preparation
Chromosome condensation
Hybridization quality
Quality of DNA-probes
Quality of probe labeling
Fluorochromes involved in an interchromosomal rearrangement
Size of the rearranged DNA-fragment

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#### High resolution multicolor banding

This technique is based on the use of differentially labeled overlapping microdissection libraries. The fluorescence intensity ratios along the chromosomes are used to assign different colors to specific chromosome regions. Using this approach, 23 bands were obtained for chromosome 5 (Chudoba et al., 1999). This technique has the potential to facilitate breakpoint mapping and it should identify peri- and paracentric inversions with ease. However, whether it is also applicable for the identification of small deletions (microdeletions) has not yet been explored.

#### Resolution and image analysis

At the beginning of the FISH era, signals were subject to visual interpretation only. Visual interpretations alone are very powerful for a number of applications. However, in order to standardize FISH signals and to automate the evaluation of FISH experiments, some principles of quantitative FISH analysis are needed. Image analysis in FISH was stimulated tremendously through the introduction of the comparative genomic hybridization (CGH) technique (Kallioniemi et al., 1992). CGH marked a new era in FISH in which many labs started with large-scale fluorescence intensity measurements on a routine basis. Instead of manual manipulation of images, sophisticated procedures were developed for an automated evaluation with accurate assessments of fluorescence intensity values and well-defined parameters for image acquisition were established (du Manoir et al., 1995, Piper et al., 1995). Similarly, many of the multi-color systems depend critically on quantitative image analysis of the fluorescence signals.

In this respect a reliable assessment of significant differences between various 24-color karyotyping systems is difficult. First comparisons of systems showed neither a winner nor a loser (Strefford et al., 2001; Rens et al., 2001). An attempt of a "mathematical proof" that the SKY system may be superior to any filter-based system (Garini et al., 1999) could be shown to be incorrect (Castleman et al., 2000). However, for most applications the most important issue will not be the system itself but rather what resolution can be achieved. Table 1 summarizes a number of factors, which have an important impact on resolution and are system independent. Additional considerations regarding resolution and which should be helpful to

select the best approach for specific applications are discussed below.

As pointed out earlier (Uhrig et al., 1999; Azofeifa et al., 2000), in each cell there is an internal control to estimate the quality of the hybridization and the resolution. The different labeling of the X and the Y chromosome in the 24-color hybridization mixes should result in additional bands on the X chromosome at the first pseudoautosomal region at Xp22.3 (size: 2.6 Mb) and the XY homology region at Xq21.3 (size: 4 Mb). The second pseudoautosomal region at Xq28 (320 kb) is never detected. Thus, each cell has a “control translocation” and “control insertion” with a unique spectral composition. Using these regions, resolution should be somewhere between 320 kb and 2.6 Mb.

However, it is important that resolution limits should in general never be given in absolute numbers. As numerous papers have shown in the past, any 24-color karyotyping system is capable of identifying very small interchromosomal aberrations. Yet, the detection of – for example – one 1.5-Mb interchromosomal rearrangement does not allow the conclusion that all possible 1.5-Mb exchanges will be detected as well. To demonstrate this in more detail, we will focus on the parameter “fluorochromes involved in an interchromosomal rearrangement” in Table 1. As shown in Fig. 4 and demonstrated earlier (Azofeifa et al., 2000) the identification of a small (~ 3 Mb or less) interchromosomal rearrangement depends critically on the fluorochrome composition of the involved chromosomes. The reason for this is that fluorescence often flares into adjacent regions and interferes with the karyotypic interpretation. For example, the identification of a small translocation is problematic if the translocated segment is only labeled with a subset of fluorochromes, which occur also in the centric segment (Fig. 4a). In this case identification of a translocation depends on the correct delineation of size differences of painting signals in different color channels. The smaller the translocation the more difficult is the detection of such a size difference. In general, interchromosomal rearrangements are much easier identified if the rearrangement results in the addition of a new fluorochrome to the derivative chromosome (Fig. 4b). Thus, small interchromosomal rearrangements may be overlooked in an otherwise perfect experiment solely because of the fluorochrome labeling of the probe mix. Therefore, depending on the fluorochrome composition some small rearrangements may be identified with ease while others with the same size may be missed.

Thus optimized probe sets, which increase the detection of all possible interchromosomal rearrangements with the best possible accuracy, should be designed. One strategy aims at labeling probes as differently as possible (Azofeifa et al., 2000). This “as different as possible” means, that each translocated or inserted segment adds at least one new fluorochrome to the derivative chromosome. This can be achieved when all chromosomes are labeled with the same number of fluorochromes. For example, if each chromosome is labeled using a double combination, eight fluorochromes would be needed as eight fluorochromes allow 28 different double combinations. As an intermediate to achieve this goal a probe set with seven fluorochromes was realized recently. In this seven-fluorochrome mix

three chromosomes were labeled with only one fluorochrome and 21 chromosomes with a double combination (for details see Azofeifa et al., 2000). The impact on classification is summarized in Fig. 4. It is obvious that this conceptual change is not only important in diagnostic applications but also in tumor cytogenetics as malignant cells may have a number of small insertions and/or translocations. This is exemplarily illustrated in Fig. 5, which depicts a seven-fluorochrome M-FISH analysis of the non-small-cell lung cancer cell line A427. Many of these rearrangements are small and could be missed or misclassified with a traditional five-fluorochrome probe set.

Discussing resolution, two issues have to be distinguished: firstly, to identify the presence of an interchromosomal rearrangement in the first place; secondly to classify it correctly. If the rearrangement is beyond a certain size, and depending on the fluorochrome composition, a correct, automated classification may be impossible. For small rearrangements, classification based on visual inspection of all individual fluorochrome channels is sometimes superior to the automated classification. The visual inspection of individual fluorochrome images is an additional tool to ensure that each chromosome segment is properly assigned and represents a special feature of filter-based systems.

Even the application of highly sophisticated image analysis does not eradicate the possibility of artifacts. For example, an additional color at the site of translocation breakpoints can be caused by the blending of colors by fluorescence flaring at the junctions of the individual chromosome painting probe domains. The size of this band depends mainly on the chromosome condensation (Azofeifa et al., 2000). Color blending also occurs at sites where two or more different chromosomes overlap in the spread. In such overlapping regions high fluorescence intensity values are observed which can create “false spectral combinations” or “nonexisting combinations”. By examining several spreads potential problems in chromosome characterization can be avoided. Some of these observations were confirmed by others, demonstrating that the same classification artifacts can be observed with different multicolor systems (Lee et al., 2001).

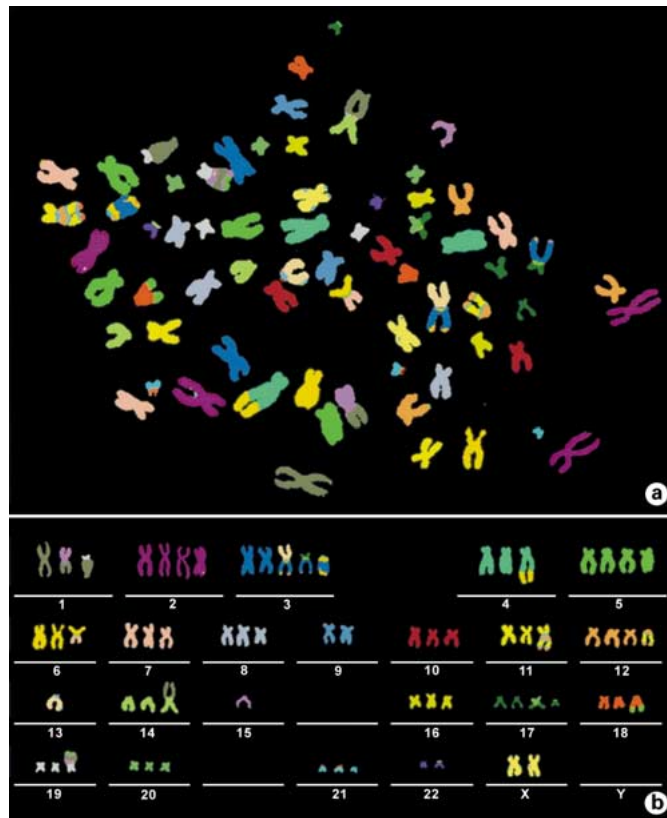
The smaller the interchromosomal aberration the more difficult it will be to distinguish it from background noise and to classify it correctly. Smoothing operations in the automated classification software can create homogeneously appearing pictures but may obscure small rearrangements. In attempts to improve the automated analysis of our imaging system it has been updated significantly over time from an algorithm based on thresholding (Speicher et al., 1996b) to an adaptive and self-calibrating spectral image analysis (Eils et al., 1998), which was more recently extended to include region specific information (Saracoglu et al., 2001).

However, even the improved 24-color karyotyping technologies cannot compete with the resolution of sophisticated telomere integrity assays for the detection of subtelomere rearrangements or with the resolution of the bar coding techniques for the elucidation of intrachromosomal aberrations. Thus, it is important to keep limitations of individual techniques in mind and to select the approach best suited for specific applications.

A	B	visual	auto	frequency (%)		
				5f	7f	8f
				13.5	3.3	0
				81.6	58.7	43.5
					4.9	38.0

4

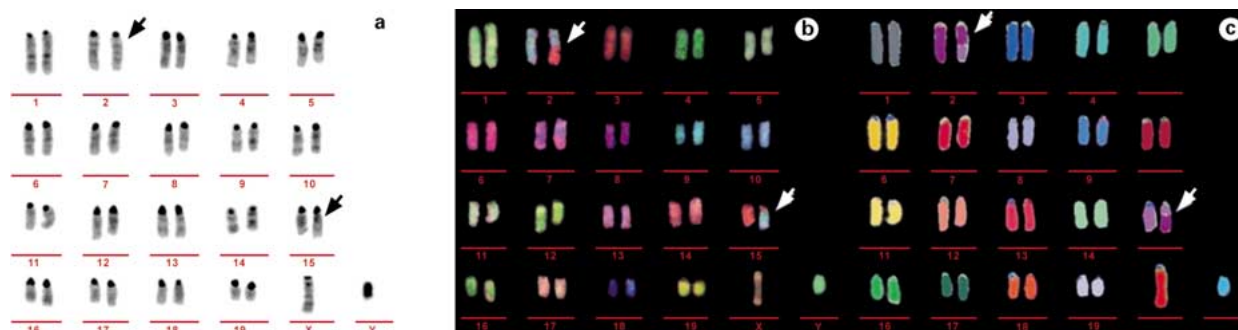
**Fig. 4.** Scheme illustrating the impact of probe design on the accurate identification and classification of small interchromosomal rearrangements. It is shown exemplarily for translocations, but the same considerations apply to small insertions as well. There are 552 possible two-way translocations in a male metaphase spread. If small (~3Mb or less), the design of the probe mix determines critically the number of translocations, which can be detected and correctly classified. **(a)** Illustration of a small translocation, which has both a low sensitivity and a low specificity. Chromosome A is labeled with two fluorochromes (red and yellow), chromosome B is labeled with the yellow fluorochrome only. If part of chromosome B is translocated to chromosome A, the translocated segment is only labeled with a fluorochrome that also occurs in the centric segment. A small translocation may be completely obscured by fluorescence flaring and hence escape both, the visual inspection of individual fluorochrome channels (visual) and the automated classification (auto). Thus, a small interchromosomal rearrangement may be overlooked simply because of the fluorochrome composition in rearranged chromosomes. With the commonly used 24-color painting kits, which employ five different fluorochromes, 13.5% out of all possible 552 translocations may be overlooked for this reason (5f). Using an optimized seven-fluorochrome probe set, only 3.3% of all possible small interchromosomal rearrangements are overlooked because of the labeling (7f). If all chromosomes are labeled each with two different fluorochromes by using eight fluorochromes, this situation is completely avoided (8f) (for details see Azofeifa et al., 2000 and text). **(b)** A small translocation is in general much easier to identify if the translocated segment adds an extra fluorochrome to the centric segment.



5

Two examples are depicted. In the first example shown in the upper row, the two chromosomes involved in the translocation have one fluorochrome in common. The translocation will be identified with high sensitivity but low specificity because fluorescence flaring may hamper distinguishing between the possible double combinations blue/yellow or blue/red. The frequencies for these high sensitivity/low specificity translocations are 81.6% (five fluorochrome mix), 58.7% (seven fluorochrome mix), and 43.5% (eight fluorochrome mix), respectively. The lower row shows an example in which both the translocated and the centric segment are labeled each with two different fluorochromes. If the analysis is done with the eight fluorochrome mix the translocation will be identified in any case unequivocally and cannot be obscured by fluorescence flaring. Translocations, which can be identified with a high specificity and sensitivity due to unique fluorochrome combinations occur with frequencies of 4.9% (5f), 38.0% (7f), and 56.5% (8f), respectively.

**Fig. 5.** A metaphase spread **(a)** and corresponding karyogram **(b)** from non-small-cell lung cancer cell line A427 after hybridization of the seven fluorochrome hybridization mix.



**Fig. 6.** An example of a mouse M-FISH experiment is shown in the inverted DAPI karyogram **(a)**, in true colors **(b)**, and in classification colors **(c)**. The karyotype is: 40,XY,t(2;15).

## Outlook

The multicolor approaches will be useful for a broad range of applications (for reviews see LeBeau 1996; Lichter 1997). Already now we have an intriguing arsenal of technologies at hand, which allow screening and analysis of both inter- and intrachromosomal alterations.

For selected applications a further increase in probe number could have advantages. For example, with both the COBRA (Wiegant et al., 2000) and the M-FISH (Karhu et al., 2001) system 42-color experiments were realized for the differential painting of all chromosome arms. The correct assignment of breakpoints is facilitated tremendously in complexly rearranged tumor metaphases by such an approach. The multicolor techniques can in addition be extended to other species. The first realization was the mouse SKY system (Liyanage et al., 1996). Its usefulness is well documented in a number of examples in which mouse karyotyping assisted in elucidating specific functions of genes (e.g. Coleman et al., 1997; Ferguson et al., 2000; Difilippantonio et al., 2000; Artandi et al., 2000). An example of mouse M-FISH (Jentsch et al., 2001) is shown in Fig. 6. Mouse and human M-FISH were recently combined for the simultaneous analysis of all chromosomes in human-mouse hybrid cells (Langer et al., 2001), representing the first simultaneous analysis of two different genomes in one hybridization. Provided that good-quality painting probes will be available for other species, automated karyotyping will be feasible for them as well.

Future developments will include specific diagnostic probe sets. For example, a centromere-specific multicolor FISH approach for the elucidation of the origin of marker chromosomes was reported recently (Nietzel et al., 2001). Other diagnostic probe sets could aim at facilitating the identification of specific chromosomal translocations in leukemia and lymphomas. If rearrangements are very complex, e.g. in tumor cytogenetics, breakpoint mapping based on fluorescence signals from painting probes and comparison with banding pattern might lead to wrong assignments. Thus, ready to use multicolor bar codes covering defined regions will be needed and will evolve to important additional tools in molecular cytogenetics. With the completion of the human genome project and the identification of more and more DNA probes (Cheung et al., 2001; McPherson et al., 2001) it is expected that specific probe sets can now be developed very rapidly. In addition, the generation of DNA

probes by microdissection (Meltzer et al., 1992) and subsequent amplification with DOP-PCR (Telenius et al., 1992) provides another indispensable probe-source.

The application of multicolor FISH to interphase cytogenetics has yet to be explored in detail. Additional experiments will be required to determine if these methods 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 analyze 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. In addition, it should be possible to assemble probe panels to address a broad spectrum of specific biological and clinical questions.

Currently, the efficient screening for unknown microdeletions with cytogenetic tools remains an unsolved problem. The emerging array/chip CGH technologies (Solinas-Toldo et al., 1997; Pinkel et al., 1998; Pollack et al., 1999) offer the potential to fill this gap (Antonarakis 2001). The array CGH technologies have the potential to compete with a number of multicolor FISH applications, such as the accurate mapping of deletions or over-represented regions. However, multicolor FISH will never be completely replaced by array CGH as the latter technology cannot identify balanced aberrations and as it is problematic in all cases of mosaicism. Moreover, the exact resolution limits of array CGH have not yet been determined.

Additional improvements may result from new probe generations, such as peptide nucleic acid probes (Taneja et al., 2001) or the use of entirely different fluorochrome classes (Tanke et al., 1998). In addition, future developments of new microscope generations and imaging systems could again have a dramatic impact on the way we will analyze chromosomes in the future.

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## References

- Arnold J: Beobachtungen über Kerntheilungen in den Zellen der Geschwülste. *Virchows Archiv* 78:279–301 (1879).
- Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, DePinho RA: Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* 406:641–645 (2000).
- Antonarakis SE: BACKing up the promises. *Nature Genet* 27:230–232 (2001).
- Azofeifa J, Fauth C, Kraus J, Maierhofer C, Langer S, Bolzer A, Reichman J, Schuffenhauer S, Speicher MR: An optimized probe set for the detection of small interchromosomal aberrations by 24-color FISH. *Am J hum Genet* 66:1684–1688 (2000).
- Brezkove V, Hansson K, van der Burg M, van der Smagt JJ, Hilhorst-Hofstee Y, Wiegant J, Beverstock GC, Raap AK, Tanke H, Breuning MH, Rosenberg C: Individuals with abnormal phenotype and normal G-banding karyotype: improvement and limitations in the diagnosis by the use of 24-colour FISH. *Hum Genet* 106:392–398 (2000).
- Brown J, Saracoglu K, Uhrig S, Speicher MR, Eils R, Kearney L: Subtelomeric chromosome rearrangements are detected using an innovative 12-colour FISH assay (M-TEL). *Nature Med* 7:497–501 (2001).
- Caspersson T, Farber S, Foley GE, Kudynowski J, Modest EJ, Simonsson E, Wagh U, Zech L: Chemical differentiation along metaphase chromosomes. *Exp Cell Res* 49:219–222 (1968).
- Caspersson T, Zech L, Johansson C: Differential banding of alkylating fluorochromes in human chromosomes. *Exp Cell Res* 60:315–319 (1970).
- Castleman KR, Eils R, Morrison L, Piper J, Saracoglu K, Schulze MA, Speicher MR: Classification Accuracy in Multiple Color Fluorescence Imaging Microscopy. *Cytometry* 41:139–147 (2000).

- Cheung VG, Nowak N, Jang W, Kirsch IR, Zhao S, Chen XN, Furey TS, Kim UJ, Kuo WL, Olivier M, Conroy J, Kasprzyk A, Massa H, Yonescu R, Sait S, Thoreen C, Snijders A, Lemyre E, Bailey JA, Bruzel A, Burrill WD, Clegg SM, Collins S, Dhani P, Friedman C, Han CS, Herrick S, Lee J, Ligon AH, Lowry S, Morley M, Narasimhan S, Osogawa K, Peng Z, Plazjer-Frick I, Quade BJ, Scott D, Sirotkin K, Thorpe AA, Gray JW, Hudson J, Pinkel D, Ried T, Rowen L, Shen-Ong GL, Strausberg RL, Birney E, Callen DF, Cheng JF, Cox DR, Doggett NA, Carter NP, Eichler EE, Haussler D, Kornberg JR, Morton CC, Albertson D, Schuler G, De Jong PJ, Trask BJ: Integration of cytogenetic landmarks into the draft sequence of the human genome. *Nature* 409:953–958 (2001).
- Chudoba I, Plesch A, Lörch T, Lemke J, Claussen U, Senger G: High resolution multicolor-banding: a new technique for refined FISH analysis of human chromosomes. *Cytogenet Cell Genet* 84:156–160 (1999).
- Coleman A, Schröck E, Weaver Z, du Manoir S, Yang F, Ferguson-Smith MA, Ried T, Janz S: Previously hidden chromosome aberrations in t(12;15)-positive BALB/c plasmacytomas uncovered by multicolor spectral karyotyping. *Cancer Res* 57:4585–4592 (1997).
- Dauwerse JG, Wiegant J, Raap AK, Breuning MH, van Ommen GJB: Multiple colors by fluorescence in situ hybridization using ratio-labelled DNA probes create a molecular karyotype. *Hum Mol Genet* 1:593–598 (1992).
- Difilippantonio MJ, Zhu J, Chen HT, Meffre E, Nussenzweig MC, Max EE, Ried T, Nussenzweig A: DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation. *Nature* 404:510–514 (2000).
- du Manoir S, Speicher MR, Joos S, Schröck E, Popp S, Döhner H, Kovacs G, Robert-Nicoud M, Lichter P, Cremer T: Detection of complete and partial chromosome gains and losses by comparative genomic in situ hybridization. *Hum Genet* 90:590–610 (1993).
- du Manoir S, Schröck E, Bentz M, Speicher MR, Joos S, Ried T, Lichter P, Cremer T: Quantitative analysis of comparative genomic hybridization. *Cytometry* 19:27–41 (1995).
- Eils R, Uhrig S, Saracoglu K, Sätzler K, Bolzer A, Petersen I, Chassery JM, Ganser M, Speicher MR: An optimized, fully automated system for fast and accurate identification of chromosomal rearrangements by multiplex-FISH (M-FISH). *Cytogenet Cell Genet* 82:160–171 (1998).
- Ferguson DO, Sekiguchi JM, Chang S, Frank KM, Gao Y, DePinho RA, Alt FW: The nonhomologous end-joining pathway of DNA repair is required for genomic stability and the suppression of translocations. *Proc natl Acad Sci, USA* 97:6630–6633 (2000).
- Flint J, Wilkie AO, Buckle VJ, Winter RM, Holland AJ, McDermid HE: The detection of subtelomeric chromosomal rearrangements in idiopathic mental retardation. *Nature Genet* 9:132–140 (1995).
- Garini Y, Gil A, Bar-Am I, Cadib D, Katzir N: Signal to noise analysis of multiple color fluorescence imaging microscopy. *Cytometry* 35:214–226 (1999).
- Granzow M, Popp S, Keller M, Holtgreve-Grez H, Brough M, Schoell B, Rauterberg-Ruland I, Hager HD, Tariverdian G, Jauch A: Multiplex FISH telomere integrity assay identifies an unbalanced cryptic translocation der (5)t(3;5)(q27;p15.3) in a family with three mentally retarded individuals. *Hum Genet* 107:51–57 (2000).
- Henegariu O, Heerema NA, Bray-Ward P, Ward DC: Colour-changing karyotyping: an alternative to M-FISH/SKY. *Nature Genet* 23:263–264 (1999).
- Henegariu O, Artan S, Grealley JM, Chen XN, Kornberg JR, Vance GH, Stubbs L, Bray-Ward P, Ward DC: Cryptic translocation identification in human and mouse using several telomeric multiplex FISH (TM-FISH) strategies. *Lab Invest* 81:483–491 (2001).
- Holinski-Feder E, Reyniers E, Uhrig S, Schoepen I, Golla A, Wauters J, Kroisel P, Bossuyt P, Rost I, Jedele K, Zierler H, Schwab S, Wildenauer D, Speicher MR, Willems PJ, Meitinger T, Kooy RF: 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 (2000).
- Hsu TC: Human and mammalian cytogenetics, a historical perspective. (Springer, New York 1979).
- Jentsch I, Adler ID, Carter NP, Speicher MR: Karyotyping mouse chromosomes by multiplex-FISH (M-FISH). *Chrom Res* 9:211–214 (2001).
- Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 258:818–821 (1992).
- Karhu R, Ahlstedt-Soini M, Bittner M, Meltzer P, Trent JM, Isola JJ: Chromosome arm-specific multicolor-FISH. *Genes Chrom Cancer* 30:105–109 (2001).
- Kingsley K, Wirth J, van der Maarel S, Freier S, Ropers HH, Haaf T: Complex FISH probes for the subtelomeric regions of all human chromosomes: comparative hybridization of CEPH YACs to chromosomes of the Old World monkey *Presbytis cristata* and great apes. *Cytogenet Cell Genet* 78:12–19 (1997).
- Knight SJL, Horsley SW, Regan R, Lawrie NM, Maher EJ, Cardy DLN, Flint J, Kearney L: Development and clinical application of an innovative fluorescence in situ hybridization technique which detects submicroscopic rearrangements involving telomeres. *Eur J hum Genet* 5:1–8 (1997).
- Knight SJL, Regan R, Nicod A, Horsley SW, Kearney L, Homfray T, Winter RM, Bolton P, Flint J: Subtle chromosomal rearrangements in children with unexplained mental retardation. *Lancet* 354:1676–1681 (1999).
- Knight SJL, Lese CM, Precht KS, Kuc J, Ning Y, Lucas S, Regan R, Brenan M, Nicod A, Lawrie NM, Cardy DLN, Nguyen H, Hudson TJ, Riethman HC, Ledbetter DH, Flint J: An optimized set of human telomere clones for studying telomere integrity and architecture. *Am J hum Genet* 67:320–333 (2000).
- Langer S, Jentsch I, Gangnus R, Yan H, Lengauer C, Speicher MR: Facilitating haplotype analysis by fully automated analysis of all chromosomes in human-mouse hybrid cell lines. *Cytogenet Cell Genet* 93:11–15 (2001).
- Lee C, Gisselsson D, Jin C, Nordgren A, Ferguson DO, Blennox E, Fletcher JA, Morton CC: Limitations of chromosome classification by multicolor karyotyping. *Am J hum Genet* 68:1043–1048 (2001).
- LeBeau MM: One FISH, two FISH, red FISH, blue FISH. *Nature Genet* 12:341–344 (1996).
- Lengauer C, Speicher MR, Popp S, Jauch A, Taniwaki M, Nagaraja R, Riethman HC, Donis-Keller H, d'Urso M, Schlessinger D, Cremer T: Chromosomal bar codes constructed by fluorescence in situ hybridization with *Alu*-PCR products of multiple YAC clones. *Hum Mol Genet* 2:505–512 (1993).
- Lichter P, Tang CC, Call K, Hermanson G, Evans GA, Housman D, Ward DC: High resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. *Science* 247:64–69 (1990).
- Lichter P: Multicolor FISHing: what's the catch. *Trends Genet* 13:475–479 (1997).
- Liyanage M, Coleman A, du Manoir S, Veldman T, McCormack S, Dickson RB, Barlow C, Wynshaw-Boris A, Janz S, Wienberg J, Ferguson-Smith MA, Schröck E, Ried T: Multicolor spectral karyotyping of mouse chromosomes. *Nature Genet* 14:312–315 (1996).
- McPherson JD, Marra M, Hillier L, Waterston RH, Chinwalla A, Wallis J, et al: A physical map of the human genome. *Nature* 409:934–941 (2001).
- Meltzer PS, Guan XY, Burgess A, Trent J: Rapid generation of region specific probes by chromosome microdissection and their application. *Nature Genet* 1:24–28 (1992).
- Moore JES, Arnold G: On the existence of permanent forms among the chromosomes of the first meiotic division in certain animals. *Proc Royal Soc London, Series B* 77:563–570 (1906).
- Morrison LE, Legator MS: Two-color ratio-coding of chromosome targets in fluorescence in situ hybridization: quantitative analysis and reproducibility. *Cytometry* 27:314–326 (1997).
- Müller S, Rocchi M, Ferguson-Smith MA, Wienberg J: Toward a multicolor chromosome bar code for the entire human karyotype by fluorescence in situ hybridization. *Hum Genet* 100:271–278 (1997).
- Müller S, O'Brien PC, Ferguson-Smith MA, Wienberg J: Cross-species colour segmenting: a novel tool in human karyotype analysis. *Cytometry* 33:445–452 (1998).
- National Institutes of Health and Institute of Molecular Medicine collaboration: A complete set of human telomeric probes and their clinical applications. *Nature Genet* 14:86–89 (1996).
- Nederlof PM, Robinson D, Abuknesha R, Wiegant J, Hopman AHN, Tanke HJ, Raap AK: Three color fluorescence in situ hybridization for the simultaneous detection of multiple nucleic acid sequences. *Cytometry* 10:20–27 (1989).
- Nederlof PM, van der Flier S, Wiegant J, Raap AK, Tanke HJ, Ploem JS, van der Ploeg M: Multiple fluorescence in situ hybridization. *Cytometry* 11:126–131 (1990).
- Nederlof PM, van der Flier S, Vrolijk J, Tanke HJ, Raap AK: Fluorescence ratio measurements of double-labeled probes for multiple in situ hybridization by digital imaging microscopy. *Cytometry* 13:839–845 (1992).
- Nietzel A, Rocchi M, Starke H, Heller A, Fiedler W, Wlodarska I, Loncarevic IF, Beensen V, Claussen U, Liehr T: A new multicolor-FISH approach for the characterization of marker chromosomes: centromere-specific multicolor-FISH (cenM-FISH). *Hum Genet* 108:199–204 (2001).
- Painter TS: Studies in mammalian spermatogenesis. 2. The spermatogenesis of man. *J Exp Zool* 37:291–336 (1923).
- Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG: High resolution analysis of DNA copy number variations using comparative genomic hybridization to microarrays. *Nature Genet* 20:207–211 (1998).
- Piper J, Rutovitz D, Sudar D, Kallioniemi A, Kallioniemi OP, Waldman FM, Gray JW, Pinkel D: Computer image analysis of comparative genomic hybridization. *Cytometry* 19:10–26 (1995).
- Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brown PO: Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nature Genet* 23:41–46 (1999).
- Popp S, Jauch A, Schindler D, Speicher MR, Lengauer C, Donis-Keller H, Riethman HC, Cremer T: 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).

- Rens W, Yang F, O'Brien PCM, Solanky N, Ferguson-Smith MA: A classification efficiency test of spectral karyotyping and multiplex fluorescence in situ hybridization: Identification of chromosome homologues between *Homo sapiens* and *Hylobates leucogenys*. *Genes Chrom Cancer* 31:65–74 (2001).
- Ried T, Baldini A, Rand TC, Ward DC: 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 (1992a).
- Ried T, Landes G, Dackowski W, Klinger K, Ward DC: 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 molec Genet* 1:307–313 (1992b).
- Saracoglu K, Brown J, Kearney L, Uhrig S, Azofeifa J, Fauth C, Speicher MR, Eils R: New concepts to improve resolution and sensitivity of molecular cytogenetic diagnostics by multicolor-FISH. *Cytometry* 44:7–15 (2001).
- Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T: Multicolor spectral karyotyping of human chromosomes. *Science* 273:494–497 (1996).
- Schröck E, Veldman T, Padilla-Nash H, Ning Y, Spurbek J, Jalal S, Shaffer LG, et al: Spectral karyotyping refines cytogenetic diagnostics of constitutional chromosomal abnormalities. *Hum Genet* 101:255–262 (1997).
- Schröck E, Padilla-Nash H: Spectral karyotyping and multicolor fluorescence in situ hybridization reveal new tumor-specific chromosomal aberrations. *Semin Hematol* 37:334–347 (2000).
- Solinas-Toldo S, Lampel S, Stilgenbauer S, Nickolenko J, Benner A, Döhner H, Cremer T, Lichter P: Matrix-based comparative genomic hybridization: biochips to screen for genomic imbalances. *Genes Chrom Cancer* 20:399–407 (1997).
- Speicher MR, Ballard SG, Ward DC: Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genet* 12:368–375 (1996a).
- Speicher MR, Ballard SG, Ward DC: Computer image analysis of combinatorial multi-fluor FISH. *Bioimaging* 4:52–64 (1996b).
- Speicher MR, Petersen S, Uhrig S, Jentsch I, Fauth C, Eils R, Petersen I: Analysis of chromosomal alterations in non-small cell lung cancer by multiplex-FISH, comparative genomic hybridization, and multicolor bar coding. *Lab Invest* 80:1031–1041 (2000).
- Speicher MR, Ward DC: The coloring of cytogenetics. *Nature Med* 2:1046–1048 (1996).
- Strefford JC, Lillington DM, Young BD, Oliver RTD: The use of multicolor fluorescence technologies in the characterization of prostate carcinoma cell lines: a comparison of multiplex fluorescence in situ hybridization and spectral karyotyping data. *Cancer Genet Cytogenet* 124:112–121 (2001).
- Taneja KL, Chavez EA, Coull J, Lansdorp PM: Multicolor fluorescence in situ hybridization with peptide nucleic acid probes for enumeration of specific chromosomes in human cells. *Genes Chrom Cancer* 30:57–63 (2001).
- Tanke HJ, De Haas RR, Sagner G, Ganser M, van Gijlswijk RPM: Use of platinum coproporphyrin and delayed luminescence imaging to extend the number of targets FISH karyotyping. *Cytometry* 33:453–459 (1998).
- Tanke HJ, Wiegant J, van Gijlswijk RP, Bezrookove V, Pattenier H, Heetebrij RJ, Talman EG, Raap AK, Vrolijk J: New strategy for multi-colour fluorescence in situ hybridisation: COBRA: COmbined Binary RAtio labelling. *Eur J hum Genet* 7:2–11 (1999).
- Telenius H, Pelmear AH, Tunnacliffe A, Carter NP, Behmel A, Ferguson-Smith MA, Nordenskjöld M, Pfragner R, Ponder BAJ: Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. *Genes Chrom Cancer* 4:257–263 (1992).
- Uhrig S, Schuffenhauer S, Fauth C, Wirtz A, Daumer-Haas C, Apacik C, Cohen M, Müller-Navia J, Cremer T, Murken J, Speicher MR: Multiplex-FISH (M-FISH) for pre- and postnatal diagnostic applications. *Am J hum Genet* 65:448–462 (1999).
- Waldeyer W: Über Karyokinese und ihre Beziehung zu den Befruchtungsvorgängen. *Arch Mikrosk Anat* 32:1–112 (1888).
- Wiegant J, Wiesmeijer CC, Hoovers JMN, Schuurin E, d'Azzo A, Vrolijk J, Tanke HJ, Raap AK: Multiple and sensitive fluorescence in situ hybridization with rhodamine-, fluorescein-, and coumarin-labeled DNAs. *Cytogenet Cell Genet* 63:73–76 (1993).
- Wiegant J, Bezrookove V, Rosenberg C, Tanke HJ, Raap AK, Zhang H, Bittner M, Trent JM, Meltzer P: Differentially painting human chromosome arms with combined binary ratio-labeling fluorescence in situ hybridization. *Genome Res* 10:861–865 (2000).