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Review

Multicolor FISH probe sets and their applications

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Summary. Multicolor fluorescence *in situ* hybridization (FISH) assays are nowadays indispensable for a precise description of complex chromosomal rearrangements. Routine application of such techniques on human chromosomes started in 1996 with the simultaneous use of all 24 human whole chromosome painting probes in multiplex-FISH (M-FISH) and spectral karyotyping (SKY). Since then different approaches for chromosomal differentiation based on multicolor-FISH (mFISH) assays have been described. Predominantly, they have been established to characterize marker chromosomes identified in conventional banding analysis. Their characterization is of high clinical impact and is the requisite condition for further molecular investigations aimed at the identification of disease-related genes. Here we present a review on the available mFISH methods including their advantages, limitations and possible applications.

Key words: Marker chromosomes, Multicolor FISH, Multicolor banding (MCB), Centromere-specific, Multicolor-FISH (cenM-FISH)

Introduction

The availability of simple, rapid and consistent methods for chromosome characterization is one of the main interests in human cytogenetics. Even though the GTG-banding (G-bands by Trypsin using Giemsa) technique is still the gold standard for all routine cytogenetic techniques, its technical restrictions are well known. As chromosome morphology combined with a black and white banding pattern are the only two parameters to be evaluated, exclusively changes within the normal pattern, size variations in a chromosomal band or the chromosome itself and changes of the centromere index can be detected (Claussen et al., 2002). Thus, the origin of additional material in a structurally altered chromosome often remains questionable. To overcome such limitations fluorescence in situ hybridization (FISH) approaches were introduced into cytogenetics in the 1980s and the new field of 'molecular cytogenetics' was born (for overview see (Chang and Mark, 1997)). However, the main progress in recent years has been the introduction of multicolor-FISH (mFISH) in molecular cytogenetics. This review focuses on the developments and progress made in this field and highlights the probe sets which have been developed for specific applications.

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Presently available multicolor-FISH (mFISH) probe sets

In this review multicolor-FISH (mFISH) is defined as the simultaneous use of at least three different ligands or fluorochromes for the specific labeling of DNA - excluding the counterstain. According to this definition the first successful mFISH experiments were done in 1989 by Nederlof and coworkers by visualizing three differently labeled nucleic acid sequences, simultaneously, in blue (amino methyl coumarin acetic acid = AMCA), red (tetramethylrhodamine isothiocyanate = TRITC) and green (fluorescein isothiocyanate = FITC). The first mFISH probe sets were put together 7 years later in 1996 (Schröck et al., 1996; Speicher et al., 1996; Yurov et al, 1996).

Whole chromosome painting mFISH probe sets

Staining of each of the 24 different human chromosomes in another color at the same time using whole chromosome libraries has been described several times throughout the last few years. Different names have been introduced for more or less the same probe sets: M-FISH (= Multiplex-FISH) (Speicher et al., 1996); SKY (= spectral karyotyping) (Schröck et al., 1996); multicolor FISH (Senger et al., 1998; Tanke et al., 1998); COBRA-FISH (= COmbined Binary RAtio labelling-FISH) (Tanke et al., 1999); or 24-color-FISH (Azofeifa et al., 2000). Four to seven different fluorescence dyes were used either for combinatorial labeling and/or ratio-labeling (see as well (Liehr and Claussen, 2002a,b)).

This basic mFISH probe set (Fig. 1) has been modified either by molecular changes in the probes themselves or by addition of supplementary probes. The so-called IPM-FISH (= IRS-PCR multiplex FISH) method uses whole chromosome painting probes which are modified by an interspersed polymerase chain reaction (IRS), which leads to a 24-color-FISH painting plus an R-band-like pattern (Aurich-Costa et al., 2001). For special questions other probes were added to the basic 24-color-FISH probe set, like single copy probes (e.g. probe for human papillomavirus (Szuhai et al., 2000, 2001; Brink et al., 2002) or subtelomeric probes (Tosi et al., 1999)), chromosome-region-specific probes (e.g. a probe for the short arm of all acrocentric chromosomes (Mrasek et al., 2001) – Fig. 1) or chromosome-arm-specific probes for all human chromosomes (42-color-FISH (Wiegant et al., 2000; Karhu et al., 2001; Brink et al., 2002; Liehr and Claussen, 2002).

mFISH banding probe sets

FISH banding probe sets are defined as "any kind of FISH technique, which provides the possibility to simultaneously characterize several chromosomal subregions smaller than a chromosome arm - excluding the short arms of the acrocentric chromosomes; FISH banding methods fitting that definition may have quite different characteristics, but share the ability to produce a DNA-specific chromosomal banding" (Liehr et al., 2002). In the following paragraphs the available mFISH banding probe sets are listed according to their quality of resolution.

1. The cross-species color banding (Rx-FISH) or Harlequin-FISH probe set (Fig. 2) provides the lowest resolution of 80-90 bands per haploid human karyotype (Müller and Wienberg, 2000). The probe set consists of flow-sorted gibbon chromosomes, which are labeled with three different fluorochromes (Müller et al., 1998). A set of 110 human-hamster somatic cell hybrids, split into two pools and labeled with two fluorochromes (Müller et al., 1997), leads, when hybridized to human chromosomes, to about 100 "bars" on each chromosome. This pattern has been called 'somatic cell hybrid-based chromosome bar code'. A combination the Rx-FISH probe set with the 110 somatic cell hybrid probes results in 160 chromosome-region-specific DNA-mediated bands in human karyotypes (Müller and Wienberg, 2000; Müller et al., 2002).

2. An approach called SCAN (= spectral color banding) has been described exemplarily for one chromosome up to present. 8 microdissection libraries were created along chromosome 10 with the aim of obtaining a banding pattern similar to the GTG-banding at the 300 band level (Kakazu et al., 2001).

3. A chromosome can be characterized as well by a specific signal pattern produced by region-specific YAC (= yeast artificial chromosomes) clones. The first attempts to label each chromosome by subregional DNA probes in different colors were performed by the groups of David Ward (Lichter et al., 1990) and Thomas Cremer (Lengauer et al., 1993). A YAC-based chromosome bar code has been especially created for chromosome 12 but not for the entire human karyotype yet (for review see (Liehr and Claussen, 2002, 2002a)). A resolution of up to 400 bands can be achieved, depending on the number of applied probes.

4. The aforementioned IPM-FISH approach (Aurich-Costa et al., 2001) can be categorized as an mFISH banding probe set, as well. A resolution of about 400 bands per haploid karyotype can be attained, dependent on the chromosome quality.

5. The high-resolution multicolor-banding (MCB) technique, based on overlapping microdissection libraries producing fluorescence profiles along the human chromosomes was described first on the example

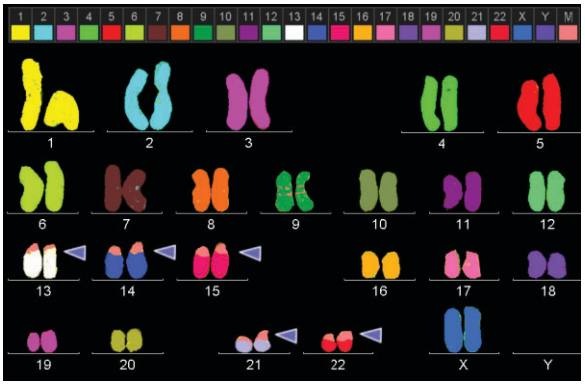

Fig. 1. 25-color FISH karyogram of a normal female metaphase (Mrasek et al., 2001). Like in M-FISH or SKY each chromosome is labeled in a different (pseudo-)color. Additionally to the 24 human whole chromosome painting probes (as it is a female no Y-chromosome is present), a probe specific for all short arms of human acrocentric chromosomes, i.e. #13, #14, #15, #21, and #22 (marked by arrowheads), is included. The probe is microdissection-derived and has been called midi54 – in the legend for the pseudocolors for each individual chromosome the 25th color for midi54 is abbreviated as "M".

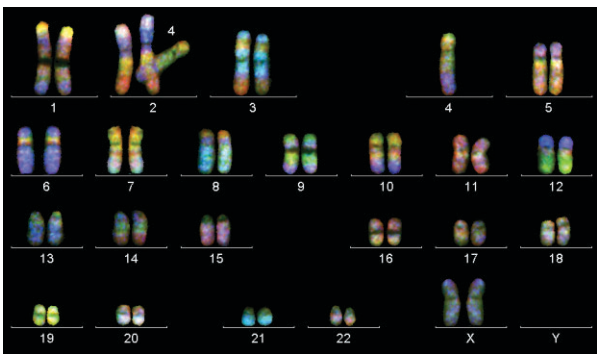

Fig. 2. Rx-FISH performed on a normal female metaphase. Chromosomes can be distinguished based on three fluorochromes and ~90 bands per haploid karyotype. However, e.g. chromosomes 21, 22 or X are not divided into subbands in that assay.

of chromosome 5 in 1999 (Chudoba et al., 1999). The so-called mBAND probe set (MetaSystems, Altlussheim, Germany) is based on the same aforementioned principle, but on slightly different microdissection derived probes than MCB. MCB/mBAND allows the differentiation of chromosome-region-specific areas at the band and sub-band level at a resolution of 550 bands per haploid karyotype. As the number of pseudo-colored bands per chromosome can freely be assigned using the isis software (MetaSystems, Altlussheim, Germany) a resolution even higher than that of GTG banding of the corresponding chromosome can be achieved, e.g. up to 10 MCB bands for chromosome 22 equals 800 bands per total haploid karyotype (Liehr et al., 2002a). Meanwhile, a complete set of approximately 140 region-specific microdissection libraries covering the entire human genome was created (Mrasek et al., 2001; Liehr et al, 2002a) (Fig. 3A). Moreover, YAC/BAC-based MCB-sets for chromosomes #2, #13 and #22 were established in parallel, which, in comparison to the microdissection-based ones, turned out to be of lower quality (Liehr et al., 2002b). On the other hand, a combination of microdissection-based MCB probe sets with locus- or breakpoint-specific probes is very promising (Weise et al., 2002). Recently, the simultaneous use of all human MCB libraries in one hybridization step for the characterization of complex karyotypes was described (see Fig. 3B (Weise et al., 2003)).

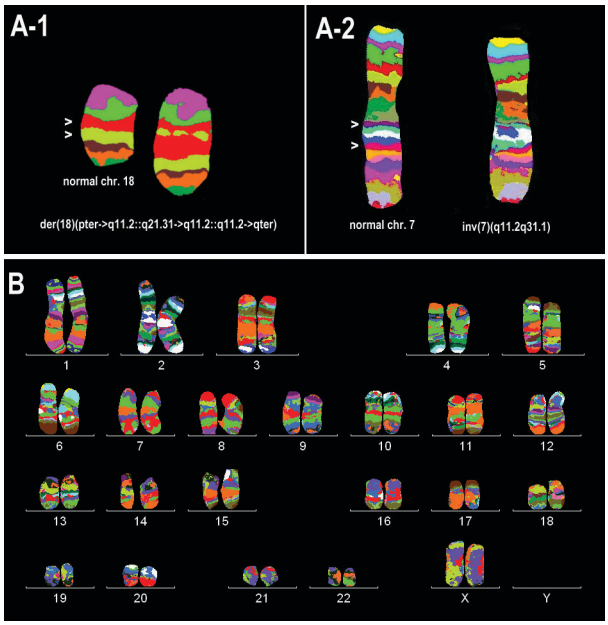

Fig. 3. Multicolor banding (MCB) results using one single chromosome-specific MCB-probe set **(A)** and all human MCB probe sets simultaneously **(B)**. The first approach is indicated when it is known which chromosomes are involved in a chromosomal rearrangement, the second, if either cryptic chromosomal changes in so-called normal karyotypes or complex karyotypes shall be analyzed. Images were captured on a Zeiss Axioplan microscope (Zeiss Jena, Germany) with the IKAROS and ISIS digital FISH imaging system (MetaSystems, Altlussheim, Germany) using an XC77 CCD camera with on-chip integration (Sony). **A.** Two examples for rearrangements resolved after application of MCB: in case A-1 an inverted duplication of 18q11.2-q21.31, and in case A-2 an inversion of 7q11.2-q31.1 in one chromosome each, were detected. The rearranged regions are marked by small arrowheads in the normal chromosomes. **B.** MCB-result on a normal female metaphase applying all MCB probes in one hybridization. In this pseudo-color depiction no optimal MCB pattern was achieved for all chromosomes simultaneously. To avoid missing rearrangements, the MCB results are evaluated not only based on the pseudo-color bands, but also based on the fluorescence profiles (for evaluation-details of MCB see Liehr et al., 2002a).

Centromere and/or locus-specific mFISH probe sets

mFISH probe sets can also be put together based on repetitive centromeric satellite or on locus-specific single-copy probes (i.e. cosmids, P1-clones, BACs, YACs). One example for this kind of probe sets is the aforementioned YAC-based chromosome bar code. Other centromere and/or locus-specific mFISH probe sets are listed below.

I. mFISH probe sets using selected centromeric probes are used widely in clinical genetics; the first mFISH approach using three different alphoid probes at the same time was described in 1996 (Yurov et al., 1996). Later, many studies reporting on the aneuploidy rate in human sperm cells e.g. after exposure to mutagens, have been published (e.g. (Rubes et al., 1998)). Another frequently studied field using three centromeric probes simultaneously is prenatal and preimplantation diagnostics using alpha satellite probes for the chromosomes X, Y and #18 (see e.g. (Harper and Wells, 1999; Thilaganathan et al., 2000)).

II. Centromere-specific multicolor FISH (cenM-FISH or CM-FISH) is a recently developed mFISH technique (Henegariu et al., 2001; Nietzel et al., 2001) which allows the simultaneous characterization of all human centromeres using labeled centromeric satellite DNA as probes. CenM-FISH distinguishes all centromeric regions apart from the evolutionary highly

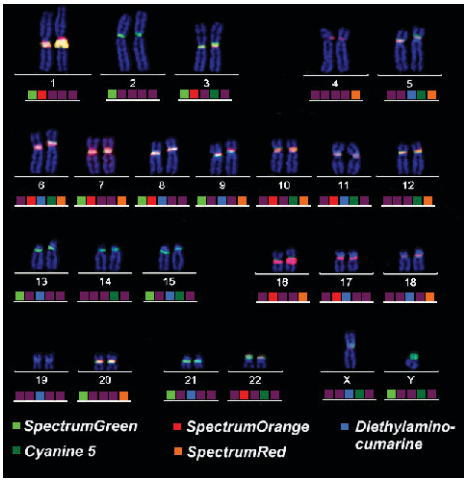

Fig. 4. Centromere-specific multicolor FISH (cenM-FISH) on a normal male metaphase (Nietzel et al., 2001). The corresponding fluorochromes applied for each human centromere are depicted below each chromosome (5 squares each).

conserved ones on chromosomes 13 and 21 in one single step by individual pseudo-coloring (see Fig. 4).

III. For the characterization of the short arms and the centromeric regions of the acrocentric chromosomes two similar probe sets are available: the acroM-FISH (Langer et al., 2001) and the acro-cenM-FISH (see Fig. 5 (Trifonov et al., 2003)) probe sets.

IV. Subcentromere-specific multicolor FISH (subcenM-FISH) is again a recently described mFISH probe set ((Starke et al., 2002) - see Fig. 6) which specifically paints a chromosomal region that not all other available FISH or mFISH probe sets can characterize: centromere near euchromatic material. This is due to the fact that these regions are either overlaid by a flaring effect of the fluorescence-intense centromeric signals, or underrepresented in other chromosome or chromosome-region-specific probes.

V. The extreme ends of all vertebrate chromosomes consist of noncoding, tandemly repeated hexanucleotide units TTAGGG (5'→3' direction), thus, the different human telomeres cannot be specifically stained using telomeric probes (Blackburn and Greider, 1995). Therefore, and as subtelomeric sequences are often underrepresented in whole chromosome painting probes, efforts have been made to develop an mFISH set consisting of subtelomeric probes (Granzow et al., 2000; Brown et al., 2001).

VI. Similar to the problems in clinical genetics which are addressed with the centromeric probes in point I, locus-specific probes were put together and are available commercially. Examples are (i) probe set kits for rapid prenatal diagnosis in uncultured amnion cells with the goal of a rapid interphase analysis for the most frequently occurring trisomies (#13, #21) (Eiben et al, 1999; Thilaganathan et al., 2000), (ii) specific mFISH assay for preimplantation diagnostics (Harper and Wells, 1999) or (iii) special multitarget mFISH for interphase tumor cytogenetics (e.g. (Sokolova et al., 2000)).

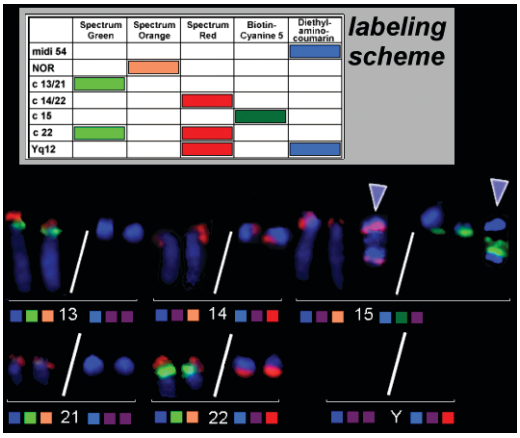

Fig. 5. Labeling scheme and result of acro-cenM-FISH in a case with a NOR-positive supernumerary marker chromosome (SMC – marked by arrowheads). The acro-cenM-FISH probe-mix contains (i) a probe specific for the acrocentric human p-arms (midi54), (ii) a NOR-specific probe (dJ1174A5), (iii) a probe specific for Yq12 (pLAY113.5), as well as (iv) the available centromere-specific probes for all human acrocentrics (Trifonov et al., 2003). The acro-cenM-FISH results allow for a description of the SMC as an idic(15)(q?12).

Applications of mFISH probe sets

The above mentioned probe sets are applied in prenatal or postnatal clinical genetics and/or tumor cytogenetics. Optimally, their use should be embedded into a strategy for the characterization of human (marker) chromosomes (Liehr and Claussen, 2002a). Nonetheless, each probe set has its own capacities and limitations, which are discussed as follows.

Whole chromosome painting mFISH probe sets have been successfully used for confirmation, refinement and/or characterization of translocations, search for cryptic rearrangements and characterization of marker chromosomes in clinical genetics, tumor cytogenetics, mutagenesis, radiobiology, evolution in mammals or interphase architecture (for overview of the corresponding literature see Liehr and Claussen, 2002a,b; Liehr, 2003). As mentioned above, the whole chromosome painting mFISH probe set can be combined with additional probes, according to the question in focus. If microdeletions (Tosi et al., 1999) or non-human DNA insertions shall be studied (Szuhai et al., 2000) single-copy probes can be added in additional color combinations. For Zoo-FISH studies it turned out to be informative to additionally introduce a probe specific for the human acrocentric chromosome p-arms (Mrasek et al., 2001).

mFISH methods using human whole chromosome painting probes reach their limits when exact breakpoint localization of translocations are required, or in case of intrachromosomal rearrangements such as interstitial deletions or inversions. Thus, different probe sets have been developed to avoid missing substantial portions of inter- and intra-chromosomal aberrations in human

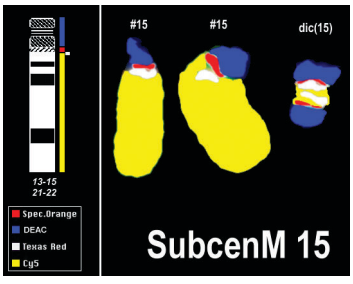

Fig. 6. Subcentromere-specific multicolor FISH (subcenM-FISH) was performed on the SMC characterized by acro-cenM-FISH in Fig. 5 – abbreviated as "dic(15)" in this figure. According to this result the SMC could be characterized as an idic(15)(q11.2-12). The applied subcenM-FISH probe set for chromosome 15 is specified in the left part of the figure: blue = midi54 (see Figs. 1 and 5); red = alpha-satellite probe for chromosome 15; white = centromere-near probe in 15q11.2 (=bA171C8); and yellow = whole chromosome paint for chromosome 15.

chromosomes. 42-color-FISH – a combination of the whole chromosome painting probes with chromosome-arm-specific probes for all human chromosomes (Wiegant et al., 2000; Karhu et al., 2001; Brink et al., 2002; Liehr and Claussen, 2002a,b) was one attempt in that direction. Though, rearrangements like paracentric inversions cannot be detected by that approach. IPM-FISH is an elegant approach connecting banding cytogenetics and M-FISH (Aurich Costa et al., 2001). However, IPM-FISH does not seem to have the potential to help to a better banding resolution when applying on condensed, contracted and highly rearranged tumor chromosomes. For the latter case the orientation of smaller chromosomal fragments can hardly be determined. The YAC-based chromosome bar code is still not available for all chromosomes (for overview see Liehr, 2003) and has the disadvantage that – per definition - it does not cover an entire chromosome but leaves gaps. This might lead to problems in exact breakpoint definition as outlined before (Liehr et al., 2002b). All FISH-banding approaches mentioned in this paragraph up to present (including SCAN (Kakazu et al., 2001)) are either incomplete, i.e. not available for the whole human karyotype, have not been extensively tested in different studies and/or are only single reports, which presented the technique as a simple idea demonstrated on few examples (overview in Liehr and Claussen, 2002).

The chromosome bar code technique using region-specific human-hamster somatic cell hybrids (Müller et al., 1997) and Rx-FISH (Müller et al., 1998) have the lowest resolution, about 80 to 100 bands per human haploid karyotype. This resolution is worse than that of chromosomes in (bad) tumorcytogenetic preparations. Moreover, the Rx-FISH bands have only seven different colors, which easily leads to ambiguous results. Thus, Rx-FISH was combined with the somatic cell hybrids using 5 different fluorochromes, which led to a higher resolution (Müller et al., 2002). Rx-FISH technology has been successfully applied in clinical, leukemia and solid tumor cytogenetics as well as in Zoo-FISH studies (overview in Liehr, 2003).

About 200 clinical cases with congenital or acquired complex chromosomal rearrangements involving different chromosomes have been studied up to now using different MCB-probe sets (overview in Liehr, 2003). In nearly all cases the results of the GTG-banding could be refined or had to be corrected. The suitability of the MCB technique to resolve complex aberrations has been proven in comparison to other techniques like CGH (Starke et al., 2001; Tönnies et al., 2001, Stumm et al., 2002), M-FISH (e.g. Houge et al., 2003; Kuechler et al., 2003; Trifonov et al., 2003), region- or locus-specific probes (Dufke et al., 2001; Starke et al., 2001a, 2002a; Liehr et al., 2002b; Trappe et al., 2002; Weise et al., 2002) and microdissection (Starke et al., 2001a,b; Heller et al., 2003) and in clinical genetics, tumor cytogenetics, mutagenesis, radiobiology, evolution in great apes or interphase architecture (overview in Liehr, 2003). As the simultaneous use of all human MCB probes is now possible (see Fig. 3B), MCB is the best available FISH-banding technique with the highest and most flexible resolution between 400 and 800 bands per haploid karyotype.

Apart from applications for special scientific approaches like the characterization of chromosomal subregions using mFISH on chromosome fibers (fiber-FISH) (Duell et al., 1997) locus-specific probes are used in the following approaches. Single probes, like cosmids, BACs, YACs and P1 clones, can either be used in combination with other mFISH approaches – like e.g. with M-FISH (Tosi et al., 1999) and with MCB to confirm breakpoint or deletion mapping (Weise et al., 2002) – or for specific clinical (e.g. Granzow et al., 2000; Brown et al., 2001) and tumor cytogenetic questions (e.g. Sokolova et al., 2000). In some of these latter approaches centromeric probes are also applied in combination with single copy probes (e.g. Eiben et al., 1999; Harper and Wells, 1999). The advantage of these probes is that they can be evaluated in metaphase and interphase. Even though the principal suitability of MCB probes for interphase cytogenetics has been demonstrated (Lemke et al., 2002), single copy and centromeric probes are the first choice for routine interphase-cytogenetics.

Probe sets with locus-specific probes for the subtelomeric (Granzow et al., 2000; Brown et al., 2001), the centromeric (cenM-FISH and CM-FISH) (Henegariu et al., 2001; Nietzel et al., 2001) and the pericentric region (Starke et al., 2002) have been developed for complementation to all the other mFISH probes for "covering the whole human karyotype". Whole chromosome painting mFISH probe sets or mFISH banding probe sets neither cover centromeric heterochromatic material of human chromosomes nor are suited to detect subtle centromere-near or telomeric aberrations. The centromeres are not visible as chromosome in situ suppression (= CISS) (Lichter et al., 1988) of labeled repetitive sequences is done and repetitive sequences present in centromeric regions of human chromosomes also become suppressed by this technique The subtelomeric regions are not covered sufficiently due to the complexity of the used probes (Granzow et al., 2000).

In up to 6% of patients with iodiopathic mental retardation cryptic subtelomeric translocations or deletions can be detected (Knight et al., 1997; Granzow et al., 2000; Brown et al., 2001), thus, efforts have been made to develop subtelomeric probes sets. In the meantime these probe sets have also identified, up to now, unknown cryptic aberrations in hematological malignancies (Brown et al., 2000). The usefulness of the cenM-FISH technique for the characterization of small supernumerary marker chromosomes (SMC) with no - or nearly no - euchromatin and restricted amounts of available sample material has been demonstrated in prenatal, postnatal and tumor cytogenetic cases (Nietzel et al., 2001, 2003; von Eggeling et al., 2002; Starke et

al., 2003). Moreover, rarely described markers with involvement of heterochromatic material inserted into homogeneously staining regions could also be identified and characterized using the cenM-FISH technique (Nietzel et al., 2001). Small SMC derived from acrocentric chromosomes (NOR-positive) can alternatively be characterized by acroM-FISH (Langer et al., 2001) or acro-cenM-FISH (Trifonov et al., 2003). The subcenM-FISH probe set was successfully applied for the characterization of the euchromatic content of small supernumerary marker chromosomes, as well as for characterization of rearranged chromosomes with involvement of centromere-near breakpoints (Starke et al., 2002a,b).

Similar to the problem addressed with the subtelomeric probe set for iodiopathic mental retardation due to cryptic subtelomeric translocations or deletions other mFISH probe sets using single-copy and/or centromeric probes have been developed for diagnostic approaches. The most important ones in clinical and tumor genetics are mentioned below:

As many microdeletion and contiguous gene-deletion syndromes include mental retardation as a clinical feature a "MultiFISH" assay has been proposed to simultaneously screen for Prader-Willi/Angelman (15q11-13), Williams-Beuren (7q11.23), Smith-Magenis (17p11.2) and DiGeorge/velocardiofacial (22q11.2) syndromes (Ligon et al., 1997). Successful redetection of 10 out of 200 patients in a blind fashion evaluation was done to prove the reliability of the technique and to exclude false positive results.

The use of mFISH techniques in uncultured amnion cells for the rapid interphase analysis of the most frequently occurring trisomies (#13, #18, #21) and numerical gonosomal aberrations is nowadays a quite often applied approach in prenatal or for preimplantation diagnostics (Eiben et al., 1999; Harper and Wells, 1999; Thilaganathan et al., 2000). Preimplantation diagnostics is especially done with the aim of detecting up to 70% of the most frequent numerical chromosome aberrations responsible for spontaneous abortions (Fung et al., 2000).

The first multitarget mFISH for interphase tumor cytogenetics was reported by Sokolova and coworkers in 2000. The detection of urothelial carcinoma cells in urine specimens is the purpose of this probe set. Such probe sets are commercially available, as well.

Conclusion

In human cytogenetics there are still various unanswered questions to study. To mention only two examples: (i) the interphase architecture is still not completely understood (review in Cremer and Cremer, 2001); or (ii) the mechanisms of marker chromosome formation, especially of SMC formation are still under discussion (Kotzot, 2002; Daniel and Malafiej, 2003). Such questions can now be addressed with recently described mFISH probe sets like MCB, cenM-FISH or subcenM-FISH. However, each approach has new abilities but also its restrictions. Thus, it is not likely that the development of new mFISH probe sets with original applications will come to an end soon. For example, all the new and exciting new possibilities with the so-called "living colors" (e.g. Nishi et al., 2002) will especially bring forward the research on the architecture of the interphase nucleus. Furthermore, combinations of the visualization of DNA in parallel to proteinstructures, like in FICTION (= fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasm) (Martin-Subero et al., 2002) will be advanced. Thus, applications not exclusively in tumor-cells, but studies on tissue-specific differences will be enabled due to such developments.

In summary, the future of mFISH approaches on human chromosomes will be mainly influenced by further technical improvements. In this context, the quality of the metaphase spreads and of spherical interphase nuclei (Steinhaeuser et al., 2002) used for FISH experiments especially needs further standardization and optimization. Better results on a rigorously reduced number of metaphase spreads needed for chromosome analysis are necessary to achieve in consequence less expensive mFISH experiments.

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