Multicolor FISH in two and three dimensions for clastogenic analyses

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Introduction

Fluorescence in situ hybridization (FISH) has evolved over the last decades to a diversely applied technology. FISH is employed in both biology and medicine for karyotype analysis, gene mapping, DNA replication and recombination, clinical diagnosis and monitoring of disease, radiation dosimetry, gene transcription and the study of chromatin organization and structure (for a detailed review see Carter, 1996). Recently, the range of FISH applications was complemented by a diversity of probes, new fluorochromes and detection systems. As a result, an increasing number of different DNA probes can be hybridized and analyzed simultaneously. This has evolved to a multitude of approaches, strategies and technologies to address different diagnostic and biological problems. Several reviews have already described the history of multicolor FISH (M-FISH) and its potential and range of applications (see for example LeBeau, 1996; Speicher and Ward, 1996; Lichter, 1997; Schröck and Padilla-Nash, 2000; Fauth and Speicher, 2001). Table I summarizes currently known multicolor FISH technologies and their preferential usage.

In particular, the multicolor karyotyping technologies M-FISH, SKY and COBRA are already widely used technologies in both diagnostic and research applications. Advantages include that karyotyping can be automated and that the resolution for the detection of small interchromosomal rearrangements is unprecedented as compared with the classical banding technologies. However, recently it has become clear that all M-FISH karyotyping technologies, besides the recognized advantages and possibilities, have pitfalls and limitations (Azofeifa et al., 2000; Lee et al., 2001; Saracoglu et al., 2001). Due to these pitfalls a substantial proportion of interchromosomal aberrations may be misclassified or may even be missed. Users of multicolor karyotyping systems should be aware of these drawbacks, which are not dependent on the system but rather a matter of the probe set and the number of different fluorochromes used to label the probes (Azofeifa et al., 2000). As a consequence, and to improve resolution considerably, we realized a conceptual change in probe labeling. This conceptual change consists of an increase in the number of fluorochromes from five to seven so that most probes are labeled with a fluorochrome double combination. Multicolor karyotyping systems employing probe sets with only five fluorochromes have a sensitivity and specificity for the detection of small (~3 Mb or less) interchromosomal rearrangements of merely 86.5 and 4.9%, respectively. In contrast, the seven fluorochrome probe mix could increase both sensitivity and specificity considerably, to 96.7 and 38.0%, respectively (Azofeifa et al., 2000).

Here, we present recent examples in which M-FISH was applied to study the effects of exposure to different agents on chromosomes. Both, human and mouse M-FISH were employed (Bardelli et al., 2001; Adler et al., 2002).

In the first example M-FISH was used in experiments designed by Christoph Lengauer (Johns Hopkins Oncology Center, Baltimore, MD). The experiments were based on a hypothesis postulated by Breivik and Gaudernack (1999). This hypothesis claimed that, depending on the selection pressures exerted on cells, different genomic instability patterns may occur in these cells. In colorectal cancer and probably in the majority of epithelial cancers the predominant genomic instability patterns are either microsatellite instability (MIN) or chromosomal instability (CIN) (Lengauer et al., 1997, 1998). Thus, Breivik and Gaudernack developed models of how either MIN or CIN may be evoked if the same parental cell line is exposed to different carcinogens.

In the MIN pathway, a methylating carcinogen, e.g. N-methyl-N-nitro-N-nitrosoguanidine (MNNG) together with DNA hypermethylation may promote G/T mismatches (Breivik and Gaudernack, 1999). As a consequence, mismatch repair should be activated and should result in growth arrest. However, under such a selection pressure mismatch repair-deficient cells should preferentially be able to surpass such a selection barrier.

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3To whom correspondence should be addressed: Institut für Humangenetik, Technische Universität München, Trogerstrasse 32, D-81675 München, Germany. Tel.: +49 89 4140 6381; Fax: +49 89 4140 6382; Email: speicher@humangenetik.med.tu-muenchen.de
as growth arrest is not invoked. Therefore, cells that overcome extended exposure to methylating carcinogens should express the MIN phenotype. As the MIN phenotype is not associated with chromosomal aberrations, such as numerical or structural aberrations (Schlegel et al., 1995), cells surviving exposure to a methylating carcinogen should show an almost identical chromosome set as the parent cells prior to exposure.

In the CIN pathway, bulky adduct forming agents, e.g. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and DNA hypomethylation may promote chromosomal rearrangements. Normal cells should activate mitosis checkpoints, which should result in growth arrest. However, cells with defects in mitosis checkpoints may have a growth advantage under such a selection pressure, so that cells with a CIN phenotype are preferentially promoted. Hence, if cells which survive such culture conditions are karyotyped, a chromosomal instability pattern would be expected.

The cell line H3, which was derived from the colorectal cancer cell line HCT 116, was used. HCT 116 is a well-known MIN colorectal cancer cell line. H3 was generated from HCT 116 by transferring a chromosome 3 into this cell line, which rendered it mismatch repair-proficient (Koi et al., 1994). In separate cultures this cell line was exposed to either MNNG or PhIP (for details see Bardelli et al., 2001). Seven fluorochrome M-FISH was applied to confirm the instability patterns observed by our colleagues at Johns Hopkins with optimal resolution at the chromosomal level. As shown in Figure 1a, cells exposed to MNNG indeed maintained a stable karyotype, which was almost identical to the parental cell line H3, whereas cells after PhIP exposure developed a CIN phenotype (Figure 1b).

In the second example, we assisted in the evaluation of induction of chromosomal aberrations by dacarbazine in germlinal cells of mice (Adler et al., 2002). Dacarbazine is a chemotherapeutic agent used to treat a variety of cancer entities. To evaluate the clastogenic effects of dacarbazine in somatic and germlinal cells male mice were injected i.p. with 500 mg/kg dacarbazine (group of Dr Adler, Institute of Experimental Genetics, GSF, Neuherberg, Germany). Later the mice were mated to untreated females of the same stock. Pregnancies were allowed to come to term. Progeny of both sexes were mated and possible translocation heterozygotes were identified by reduced fertility (for details see Adler et al., 2002). Suspected translocation carriers were subjected to cytogenetic confirmation. However, karyotyping of mouse chromosomes is a skillful art, which is laborious even for experienced cytogeneticists. This is because mouse chromosomes are not characterized by significant size differences and, as all of them are acrocentric, they lack many of the landmarks which facilitate the identification of human chromosomes. We have used our mouse M-FISH technology (Jentsch et al., 2001) to identify translocations unequivocally. Some of the results have been published previously (Jentsch et al., 2001; Adler et al., 2002). Figure 2 shows an as yet unpublished karyogram of this study with an additional copy of the X chromosome. This example demonstrates that M-FISH technologies facilitate the analysis of chromosomes tremendously. Even chromosomes of distant species are now amenable to high resolution analysis.

Multicolor karyotyping in particular is advantageous in cases of multiple and complex rearrangements. However, depending on the problem to be addressed, the simultaneous hybridization of a multitude of region-specific probes may be better suited. Due to the human genome project there is a growing number of BAC clones available which have been characterized both cytogenetically and on the molecular genetic level (Cheung et al., 2001). Easy to use Internet web pages such as the Human Genome Browser from the Wellcome Trust Sanger Institute (http://www.ensembl.org/) facilitate the selection of BAC probes. Painting probes, arm-specific probes, centromere-specific probes or other probes can be obtained from the University of Bari, Italy (http://www.biologia.uniba.it/rmc/index.html). With these resources, available research groups

Table I. Currently existing multicolor-FISH technologies and their main applications

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Multicolor FISH for clastogenic analyses

Fig. 1. A cell line (H3) derived from the colorectal cell line HCT 116 was exposed either to the methylating agent MNNG or to the bulky adduct-forming agent PhIP. Seven fluorochrome M-FISH was applied to surviving clones to study the effects of these carcinogens on chromosomes. (a) A metaphase from a clone which survived exposure to MNNG. All analyzed metaphase spreads resembled the parent clone, thus, no cytogenetically visible changes were induced by MNNG. (b) Seven fluorochrome M-FISH analysis of a clone from a metaphase spread after exposure to PhIP. A number of structural and numerical changes were observed, which did not occur in the parental line. In addition, a considerable variability from one metaphase spread to the other was observed. For details see text or Bardelli et al. (2001).

should be able to tailor probe sets to their specific needs. This is further facilitated by a growing number of spectrally resolvable fluorochromes which cover the entire spectrum from the UV to the far infrared range. Thus, chromosomes are now amenable to a detailed analysis with unprecedented resolution.

The accurate assessment of instability depends on the analysis of relatively large cell numbers. This is because instability is defined as a matter of rate (Lengauer et al., 1998). In contrast, the existence of a genetic alteration by itself does not indicate an instability, as it provides no information about the rate of its occurrence. Therefore, interphase cytogenetics is considered to be the most efficient molecular cytogenetic tool to assess the instability rate. Recent experiments have shown that the application of M-FISH to interphase cytogenetics is feasible. M-FISH can be used effectively to enumerate chromosomes in intact cells or tissues (Figure 3). To study cells within their natural tissue context, we used algorithms for deconvoluting optical section images recorded by CCD cameras. Deconvolution refers to a computational method used to reduce out of focus fluorescence in 3-dimensional microscopy images (Carrington et al., 1995). After deconvolu-

Fig. 2. Mouse M-FISH analysis of mouse DAC-1049, which has an additional X-chromosome. For details see text or Adler et al. (2002).

Fig. 3. 20 µm thick breast cancer specimen after hybridization with a five color centromere-specific probe set. The probe set consists of probes for chromosomes 7 (pink), 8 (green), 11 (blue), 17 (yellow) and 18 (red). For a detailed analysis sophisticated 3-dimensional reconstruction technologies using deconvolution were developed.
further details will be published elsewhere (C. Maierhofer et al., manuscript in preparation).

In summary, FISH has boomed considerably in the last few years. In part, this upsurge was caused by efficient tools for M-FISH as it has broadened the range of applications considerably. A major advantage of FISH is its potential for single cell analysis which makes it an indispensable tool whenever aberrations which occur in only a subset of cells have to be unraveled. The molecular cytogenetic toolbox has recently been further complemented by array/matrix CGH technologies (Solinas-Toldo et al., 1997; Pinkel et al., 1998). Genome-wide array CGH for human (Snijders et al., 2001; Cai et al., 2002) and mouse (Hodgson et al., 2001) and arrays specifically designed for certain disease entities (Fritz et al., 2002; Veltman et al., 2002; Wessendorf et al., 2002; Wilhelm et al., 2002) have been reported. Furthermore, several PCR amplification protocols for single cell CGH were published recently (Klein et al., 1999; Vouillaire et al., 1999; Wells et al., 1999). The robustness of these protocols is currently being tested, mainly in preimplantation applications (Vouillaire et al., 2000; Wells and Delhanty, 2000; Malmgren et al., 2002). No experience exists as yet of whether these single cell PCR protocols yield reliable results if applied to arrays, but, without doubt, this will be tested in the near future. Even the possibility of a combined transcriptome and genome analysis at the single cell level has been published (Klein et al., 2002), indicating that a wealth of information can be extracted from a single cell. In terms of further developments, there is no end in sight. New probe generations, such as peptide nucleic acid probes (Taneja et al., 2001), new detection systems, such as rolling circle amplification (Lizard et al., 1998; Zhong et al., 2001), and new fluorescent probes, such as semiconductor nanocrystals (Bruchez et al., 1998; Lacoete et al., 2000), are waiting to be fully admitted to the constantly expanding arsenal of molecular cytogenetic methods. Thus, it will be exciting to see what new approaches and ideas will be realized in the future.

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