Modern molecular cytogenetic techniques in genetic diagnostics

Holger Tönnies

During the past decade, fluorescence in situ hybridization (FISH) has become an important complementing application in genetic diagnostics. The use of variable FISH techniques enhances the thorough interpretation of numerical and complex chromosome aberrations, bridging the gap between conventional chromosome banding analysis and molecular genetic DNA studies. This review gives a brief overview of the different molecular cytogenetic FISH techniques and applications currently used in routine genetic diagnostics and focus on their advantages and limitations.

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Conventional cytogenetic karyotype analysis is still the standard test in genetic diagnostics to identify numerical and structural chromosomal aberrations, which are the major cause of mental and psychomotoric retardation, miscarriages, congenital anomalies, and also common findings in neoplasia. Aberrant cytogenetic findings relevant for diagnosis, clinical management, and appropriate genetic counseling have contributed enormously to the definition of phenotype–karyotype correlations in chromosomal syndromes and hematological diseases. However, the limited chromosome specific banding resolution obtained by Giemsa banding (GTG-banding) makes the recognition and interpretation of masked or cryptic chromosome aberrations difficult to ascertain and is therefore potentially inaccurate.

We want to see more - in situ hybridization

In the late 1960s, Pardue and Gall [1] first described the in situ hybridization (ISH) of radioactively labeled repetitive DNA probes on cytological preparations. In 1986, Pinkel et al. [2] and Cremer et al. [3] reported fluorescence in situ hybridization (FISH) using non-radioactively labeled probes. FISH is based on the hybridization of complementary, single-stranded nucleic acids to fixed target genetic material (i.e. metaphase chromosomes or interphase nuclei). FISH probes are labeled either directly using fluorochrome-conjugated nucleotides (e.g. fluorescein-dUTP) or indirectly using reporter molecules (e.g. biotin-dUTP) by nick-translation, random priming, PCR, or various other molecular genetic techniques. By this sensitive method, nucleic acid probes allow the exploration of the presence, number and distinct location (in situ) of genetic material by forming a new DNA duplex with the complementary target material. Fluorescent signals of bound probes are inspected using a filter-equipped epifluorescence microscope and computer software.

Which probe for which problem?

A large number of different probes designed to identify specific chromosomes and parts thereof are available for diagnostic purposes (Table 1). The choice of probe and the simultaneous use of multi-probe essays depends on the particular application in question.

For chromosome enumeration, probes detecting chromosome-specific α-satellite regions near the centromeres are preferred (centromeric probe (cep)) (Fig 1b). For interphase cell analysis, which can be performed in cytological preparations as well as in sections of formaldehyde-fixed and paraffin-embedded tissues, these probes are particularly attractive because analysis is rapid and sensitivity is high. However, by using these probes for interphase cytogenetics, centromeric polymorphisms can result in false positive or false negative results. Recently, an all-human centromere-specific multicolour-FISH approach (cenM-FISH) for the ascertainment of the origin of structurally abnormal, cytogenetically unidentifiable chromosomes – so-called marker chromosomes – was reported by Nietzel et al. [4]. This technique allows the simultaneous characterization of all human centromeres using differently labeled centromeric satellite DNA as probes. However, by using cep probes, only the centromeric parts of chromosomes can be explored, so that other chromosome abnormalities affecting the euchromatin, are excluded from investigation.

Chromosome painting probes [whole chromosome paint (wcp) and partial chromosome paint (pcp)] obtained by flow sorting or micromanipulator-mediated chromosome microdissection, allow the labeling of individual chromosomes in metaphase spreads and the identification of both numerical and interchromosomal structural rearrangements (Fig. 1a). The use of chromosome paints to ascertain chromosomal aberrations needs prior knowledge of the affected chromosome(s) in question. However, a locus-specific determination of the affected chromosomal region in intrachromosomal rearrangements such as deletions, duplications and paraacentric inversions, and the detection of interchromosomal translocations involving only small regions of the chromosome ends (less well represented in these probes), cannot be performed successfully by using these probes.

Appropriate region-specific cloned probes [e.g. yeast artificial chromosome (YACs), bacterial artificial chromosome (BACs) and P1 artificial chromosome (PACs)] are used for the determination and characterization of intra- and interchromosomal rearrangements regardless of their complexity (Fig. 1c).

For the investigation of specific submicroscopic chromosomal regions, a wide spectrum of so-called locus-specific identifiers (LSI) are commercially available for the detection of the classical microdeletion syndromes (contiguous gene syndromes) or specific chromosome translocations followed by chimeric gene fusion common in hematological diseases (e.g. BCR/ABL). Special subtelomeric probes were established to scan metaphase spreads for cryptic aberrations at the gene-rich ends of the chromosomes, probably accounting for 5–10% of unexplained moderate-to-severe mental retardation cases [5]. Recently,
### Table 1. Molecular cytogenetic techniques used in genetic diagnostics

<table>
<thead>
<tr>
<th>Technique</th>
<th>Probe used</th>
<th>Hybridization target</th>
<th>Diagnostic applications</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH</td>
<td>Cep¹</td>
<td>Specimen metaphase spreads or interphase nuclei</td>
<td>Chromosome enumeration, marker chromosome identification</td>
<td>Simple setup and rapid results¹, no need for specific cytogenetic expertise, applicable on archived material</td>
<td>Small spectrum of analysis¹, no information about euchromatic content in marker chromosomes</td>
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<td></td>
<td>Wcp and pcp</td>
<td>Specimen metaphase spreads</td>
<td>Interchromosomal aberrations (translocations) and chromosome enumeration</td>
<td>Simple setup and fast detection of interchromosomal aberrations in question</td>
<td>Small spectrum of analysis, need for prior knowledge of affected chromosome, locus-specific determination of intrachromosomal aberrations not possible, need for proliferating material, subtelomeric regions underrepresented</td>
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<td></td>
<td>Cloned probes as YAC/BAC/PAC etc. b</td>
<td>Specimen metaphase spreads or interphase nuclei</td>
<td>Inter- and intrachromosomal aberrations [translocations, inversions, microdeletions and microduplications, chimeric gene fusions (leukemias)]</td>
<td>High sensitivity and specificity, detection of gene amplification possible (e.g. oncogenes)</td>
<td>Small spectrum of analysis, need of prior knowledge of affected chromosome locus or genes</td>
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<td></td>
<td>Subtelomeric probes c</td>
<td>Specimen metaphase spreads</td>
<td>Cryptic subtelomeric rearrangements</td>
<td>High sensitivity and specificity</td>
<td>Small euchromatic spectrum of analysis, proliferating material</td>
</tr>
<tr>
<td>Micro-FISH</td>
<td>Microdissected chromosomes or parts thereof</td>
<td>Specimen and control metaphase spreads</td>
<td>Marker chromosome characterization by forward (specimen) and reverse (control) painting</td>
<td>Straightforward and complete euchromatic characterization of chromosome in question, especially of marker chromosomes (mosaics)</td>
<td>Technically demanding, need for excellent cytogenetic expertise for chromosome dissection</td>
</tr>
<tr>
<td>Chromosomal bar codes</td>
<td>YAC clones, fragment hybrids</td>
<td>Specimen metaphase spreads</td>
<td>Inter- and intrachromosomal aberrations</td>
<td>Metaphase wide detection of gross chromosome alterations</td>
<td>Poor resolution, insensitive locus-specific determination of interchromosomal aberrations and break-point detection, staining gaps</td>
</tr>
<tr>
<td>Rx-FISH</td>
<td>Cross-species wcp-mix</td>
<td>Specimen metaphase spreads</td>
<td>Inter- and intrachromosomal aberrations</td>
<td>Metaphase wide detection of gross chromosome alterations</td>
<td>Poor resolution, insensitive locus specific determination of interchromosomal aberrations and break-point detection</td>
</tr>
<tr>
<td>Multicolor-banding (MCB)</td>
<td>Region-specific probes of one chromosome</td>
<td>Specimen metaphase spreads</td>
<td>Infracromosomal aberrations</td>
<td>Sensitive detection of inversion breakpoints, locus specific information (chromosomal band), high reproducibility</td>
<td>Technically demanding, costs per chromosome, information only about the chromosome probes hybridized</td>
</tr>
<tr>
<td>SKY/M-FISH</td>
<td>All human wcp-mix</td>
<td>Specimen metaphase spreads</td>
<td>Infracromosomal aberrations as translocations, chromosome enumeration, marker chromosome identification</td>
<td>Whole-metaphase scanning, no need for prior knowledge of chromosomes affected, sensitive translocation and marker chromosome identification</td>
<td>Need for proliferating material, insensitive locus-specific determination of intrachromosomal aberrations and break-point detection, costs, technically demanding</td>
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<tr>
<td>CGH</td>
<td>Specimen DNA</td>
<td>Control metaphase spreads</td>
<td>Chromosomal and genetic inter-, and intrachromosomal imbalances gene amplifications, marker chromosome characterization</td>
<td>Whole genome scanning technique, no need of proliferating material, applicable on archived material, detection of gene amplification, locus-specific information of imbalances (chromosomal band)</td>
<td>No detection of balanced aberrations or small imbalances, need of good cytogenetic expertise for karyotyping, missing of small mosaics</td>
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<tr>
<td>Matrix-CGH</td>
<td>Specimen DNA</td>
<td>Defined DNA probes</td>
<td>Chromosomal and genetic inter- and intrachromosomal imbalances, gene amplifications, marker chromosome characterization</td>
<td>Scanning of a large informative probe-set, individual array construction, no need for proliferating material, sensitive detection of imbalances, no cytogenetic expertise, automated signal interpretation</td>
<td>No detection of balanced aberrations or small imbalances not displayed by the probe set, missing of small mosaics, costs, technically demanding</td>
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Abbreviations: BAC, bacterial artificial chromosome; cep, centromeric probe; cenM-FISH, centromere-specific multicolour-FISH; CGH, comparative genomic hybridization; FISH, fluorescent in situ hybridization; LSI, locus-specific identifiers; M-FISH, multicolor FISH; PAC, P1 artificial chromosome; pcp, partial chromosome paint; Rx, cross species; SKY, spectral karyotyping; TM-FISH, telomeric multicolour FISH; wcp, whole chromosome paint; YAC, yeast artificial chromosome.

¹including cenM-FISH

²including commercially available LSI probes like bcr-abl and microdeletion probes.

³including TM-FISH
Henegariu et al. [6] reported the analysis of 41 chromosome ends simultaneously using a multicolour hybridization assay including DNA probes located near the end of these chromosomes (0.1–1 Mb from the telomere) (TM-FISH).

**FISH probes: make your own one**

In addition to the use of specific commercial or non-commercial cloned probes, micro-manipulated chromosome microdissection, followed by PCR-mediated DNA amplification, labeling and reverse (on normal metaphase spread) or forward (on affected cell) painting is a straightforward, highly informative strategy to characterize marker and other aberrant chromosomes (micro-FISH [7]). However, there are practical limitations to micro-FISH for diagnostic purposes as specialized equipment, technical skill, and a profound cytogenetic knowledge are necessary.

**Colourful chromosome world**

The age of multicolour FISH (M-FISH)-based chromosome banding techniques started with chromosomal bar codes [8,9] and cross-species chromosome painting (Rx-FISH [10]) covering all human chromosomes in one experiment providing new possibilities for the identification of gross intra- and interchromosomal aberrations. However, the spatial resolution of ~100 bands per haploid chromosome set is low in comparison with standard GTG-banding (~400 bands) and makes these techniques less useful for primary diagnostic screening purposes. Another recently developed single chromosome multicolour-banding (MCB) technique allows a higher resolution independent of chromosome condensation [11,12]. This technique, based on the hybridization of differently labeled overlapping microdissection probes followed by computer-based assignment of distinctive pseudo-colours, can be preferentially used for the detection of intrachromosomal aberrations as inversions and deletions. To date, this technique is limited to a single chromosome pair at a time and thus needs the prior knowledge of the chromosome affected.

**Metaphase and genome scanning techniques**

Today, among the various locus- and chromosome-specific assays described here, which often require the hybridization of a whole repertoire of different DNA probes to narrow down the overall composition of a chromosome aberration, some unbiased whole-metaphase or genome scanning approaches are established for the characterization of highly rearranged and unbalanced chromosome aberrations. Spectral karyotyping (SKY [13]) using interferometer-based spectral imaging and multicolour-FISH [14] using different fluorochrome-specific filter sets are based on the simultaneously hybridization of 24 differently labeled chromosome painting probes on non-overlapping target metaphase spreads of the specimen. By the use of computer-generated false-colour chromosome images, non-homologous structural and numerical chromosomal aberrations can be easily detected in single metaphase spreads. The main advantage of both techniques is that no prior knowledge of affected chromosome(s) is required. These techniques are capable of detecting chromosome rearrangements such as translocations and euchromatic marker chromosomes in a single hybridization, even if the chromosome morphology is poor as in solid tumours. The major drawback for routine use is the poor sensitivity to detect aberrations like intrachromosomal deletions, duplications and inversions. The large number of probes, labeled with different fluorochromes, and the special technical equipment required, in particular for SKY analyses, restrict these scanning techniques to some specialized laboratories.

**Cytogenetics on DNA: how does that work?**

Comparative genomic hybridization (CGH) [15] is a scanning technique based on the use of genomic DNA as a probe (Fig. 2). CGH is a potent and reliable hybridization approach, which allows the comprehensive analysis of the entire genome in just one experiment providing information not only about the size of all chromosomal imbalances detected but also about their chromosomal band-specific assignment. CGH is based on the cohybridization of labeled whole genomic test and control DNA in a ratio of 1:1 on normal control metaphase spreads under conditions of in situ suppression hybridization (CISS). After image capturing and karyotyping, quantification of fluorescence intensities over the entire length of each chromosome is performed by computer software calculating a ‘copy-number karyotype’ reflecting chromosomal imbalances (Fig. 2). In contrast to other primary scanning techniques such as M-FISH and SKY, no proliferating material of the specimen is necessary for screening. This allows to study any specimen for which DNA is available (e.g. paraffin-embedded tissue sections) for chromosomal imbalances on a band-specific cytogenetic level (~10 Mb). For example, CGH-based investigation of supernumerary euchromatic marker...
chromosomes provides information, not only on the chromosomal assignment but also on the chromosomal band-specific origin. Nevertheless, CGH cannot reveal balanced chromosomal rearrangements, often found in routine tumour genetics. Furthermore, by using whole genomic DNA, CGH is not sufficiently sensitive to detect aberrations occurring in a small amount of cells reflecting a cell-to-cell heterogeneity (mosaic), a characteristic feature of carcinomas and leukemias.

Nevertheless, these FISH-based scanning techniques, nowadays also utilized in pre- and postnatal diagnostics, are excellent pre-informative tools for the detection of chromosomal alterations, later to be further characterized by more sensitive, complementary methods using locus-specific probes [16–18].

**Future developments**

Recent advances, such as improved hybridization protocols and strategies, novel commercial and non-commercial probe sets, alongside the efforts of the Human Genome Project, coupled with hardware development such as image analysis software and specific filter sets for newly established fluorochromes, facilitate the collection of new important data for the understanding of genetic diseases. Other current research-based applications, too numerous to be listed in this paper, are likely to translate onto the growing list of clinically useful applications in the near future (for overview see Ref. [19]). For diagnostic purposes, new time- and cost-saving approaches with the potential to investigate high numbers of genetic loci in an automated fashion and with high resolution independently of cell culture and chromosome preparations are favoured. First steps in this direction are now being taken. In 1997, Solinas-Toldo and co-workers established a matrix-based CGH array (Matrix-CGH [20]) that replaces condensed metaphase chromosomes by well-defined cloned DNA probes immobilized on a glass surface as hybridization target, allowing automated analysis of chromosomal imbalances such as microdeletions and overrepresentations. Finally, by using whole genomic DNA, CGH is not sufficiently sensitive to detect aberrations occurring in a small amount of cells reflecting a cell-to-cell heterogeneity (mosaic), a characteristic feature of carcinomas and leukemias.

Nevertheless, these FISH-based scanning techniques, nowadays also utilized in pre- and postnatal diagnostics, are excellent pre-informative tools for the detection of chromosomal alterations, later to be further characterized by more sensitive, complementary methods using locus-specific probes [16–18].

**Fig. 2.** Comparative genomic hybridization (CGH) and matrix-comparative genomic hybridization procedures. (a) Differentially labeled DNAs are hybridized on control metaphase spreads. Copy-number aberrations are detected by measuring fluorescence intensity differences depicted in a copy-number karyotype. (b) Labeled DNAs are hybridized on defined spotted DNA clones reflecting the human genome or parts thereof. The resulting relative fluorescence intensities are measured by computer scanning. Abbreviations: Cot-1, highly repetitive human DNA; CGH, comparative genomic hybridization; DIM, diminished (deletion); ENH, enhanced (duplication/amplification).

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**References**

High-throughput protein arrays: prospects for molecular diagnostics

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High-throughput protein arrays allow the miniaturized and parallel analysis of large numbers of diagnostic markers in complex samples. Using automated colony picking and gridding, cDNA or antibody libraries can be expressed and screened as clone arrays. Protein microarrays are constructed from recombinantly expressed, purified, and yet functional proteins, entailing a range of optimized expression systems. Antibody microarrays are becoming a robust format for expression profiling of whole genomes. Alternative systems, such as aptamer, PROfusion®, nano- and microfluidic arrays are all at proof-of-concept stage. Differential protein profiles have been used as molecular diagnostics for cancer and autoimmune diseases and might ultimately be applied to screening of high-risk and general populations.

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Protein arrays appear to be promising tools for drug screening and diagnostics [1]. The concept of the arrayed library allows gene expression analysis and protein interaction screening on a whole-genome scale [2]. In contrast to DNA arrays, gene action can be studied directly, providing the proteins’ natural shape and functionality are maintained. This requires novel systems for high-throughput protein expression, which produce sufficient amounts of properly modified and folded molecules. Large numbers of proteins must be arrayed at high density, keeping them intact and biologically active. This is most easily achieved if molecules of the same general structure (e.g. antibodies) are arrayed. Antibody arrays are now becoming an important screening tool for a wide range of molecules in complex mixtures (e.g. body fluids). Alternatively, nucleotide aptamers might be able to mimic certain protein functions and make even more robust array formats in the future. Here we discuss the main technological challenges, recent progress and diagnostic prospects of high-throughput protein arrays (Table 1), a field that is still largely experimental but rapidly progressing.

Protein features and expression systems

Proteins constitute a large variety of shapes and features that govern their functions. Three-dimensional protein structures are determined by amino-acid sequences and environmental conditions. Post-translational modifications influence binding and function of many eukaryotic proteins. Glycosylation affects solubility, lifetime and activity of some proteins, such as antibodies’ ability to bind complement [3]. Phosphorylation regulates enzyme activities and allows specific protein–protein interactions via domains recognizing phosphate groups (e.g. SH-2 domains that interact with phosphorytoserine groups in signal transduction [4]). Such post-translational modifications depend strongly on the expressed protein’s cellular host. If recombinant proteins are expressed in bacteria, they are usually not glycosylated or phosphorylated, whereas S. cerevisiae tends to hyperglycosylate secreted mammalian proteins. Targeting of recombinant proteins to secretion compartments, such as the endoplasmic reticulum, allows them to form stabilizing disulfide bridges. Globular proteins consist of a hydrophilic exterior and a hydrophobic interior. If exposed to hydrophobic surfaces, organic solvents or detergents, the inside is turned out, thus rendering these proteins inactive. This emphasizes the imperative of proper handling of native proteins for functional assays and three-dimensional interaction studies. Refolding denatured proteins in vitro is an arduous task, requiring customized protocols and is not attainable in many cases.

Protein expression systems should be chosen carefully. E. coli is still the most convenient host organism, but many eukaryotic proteins end up in cytoplasmic...