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Demystified . . .

FISH

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The acronym FISH has been coined to describe the extremely powerful technique of fluorescence in situ hybridisation that has an ever increasing range of applications in medicine and biology. The technique allows the visualisation of quantitative genetic alterations on a cell by cell basis. This review attempts to establish the principles of the technique and to convey some idea of its wide applicability to many different areas of pathology. Reference will be made to more detailed technical reviews and research papers on particular applications or refinements. This review highlights the impact of FISH from the perspective of work in clinical cytogenetics.

Classic methods for the identification of human chromosomes rely on the use of various chemical stains. These are robust techniques, widely used in clinical laboratories.[1] However, they fail to allow identification of derivative (marker) chromosomes and small translocation, insertion or microdeletion events. Equally importantly, the classic techniques are applicable only to metaphase chromosomes and are critically dependent on the quality of the preparations obtained. This is particularly restricting in cytogenetic studies of leukaemias and other malignancies where preparations from marrow samples are invariably of poor quality.[2] Metaphase spreads may be obtained only from a tissue that has an intrinsically high mitotic activity (for example, first trimester chorionic villi)[3] or is cultured in vitro from a suitable tissue (for example, fresh skin biopsy)[3] for several days so that at a suitable point metaphases can be obtained from cells in division.

FISH not only allows accurate chromosome identification from poor quality preparations but permits cytogeneticists to cross the Rubicon from examination of metaphases to examination of resting cells in interphase: a much larger and potentially more informative population.

Principles of the FISH technique

FLUORESCENCE

Early work on the detection of probes relied on the use of radiolabels as reporters for sites of successful hybridisation. The radiolabels were either high energy γ emitters with a short half life and limited resolution (for example, conjugated I^{125}), or more stable, low energy β emitters (for example, conjugated H^3 (tritium)). These had greater resolution but required exposure times of several weeks. Despite these limitations, pioneering work was performed by Pardue and Gall,[4] using radiolabelled highly repetitive DNA to locate satellite III, pericentromeric sequences, on mouse chromosomes. Later Harper and colleagues[5] and Malcolm and colleagues[6] successfully hybridised single copy genes to human chromosomes and thereby were able to map the respective genes to specific chromosome loci. Advances in the ability to characterise DNA sequences of interest and package them in suitable vectors gradually increased probe availability.

As the chemistry of non-radioactive labelling reporting systems has become better understood, the use of fluor-labelled, biotin-avidin detection systems and immunodetection systems (for example, antidigoxygenin) in particular, have become well established in cytogenetics.[7] Alternative non-radioactive methods of labelling have also received attention[8] but are not widely used for this area. These labelling techniques offered the advantages of safety, speed, and efficiency together with relative stability. The use of different coloured fluor labels for each probe opened up the prospect of the simultaneous detection of a number of different probes on the same preparation. Such probes were then successfully applied to interphase cells.[9] A useful development was the ability to "flow sort" human chromosomes and then, after suitable packaging and labelling, to use chromosome specific cocktails of probes to identify a particular chromosome or combinations of chromosomes.[10] Parallel advances in microscope optics and computer aided, image processing, analysis, and storage, resulted in easier handling of data.

IN SITU

In situ techniques (for general reviews see references[11–13]) allow specific nucleic acid sequences or proteins to be detected in morphologically preserved chromosomes, cells or tissue sections. Using a suitable reporter molecule (for example, a fluorochrome) the technique is capable of generating microscope images with information on the presence of:

- a particular gene sequence (or combination of sequences) at the DNA level (the subject of this review)
- the product of gene expression at the mRNA or protein level.

Successful in situ hybridisation requires attention to detail in four areas:

- sample/slide preparation

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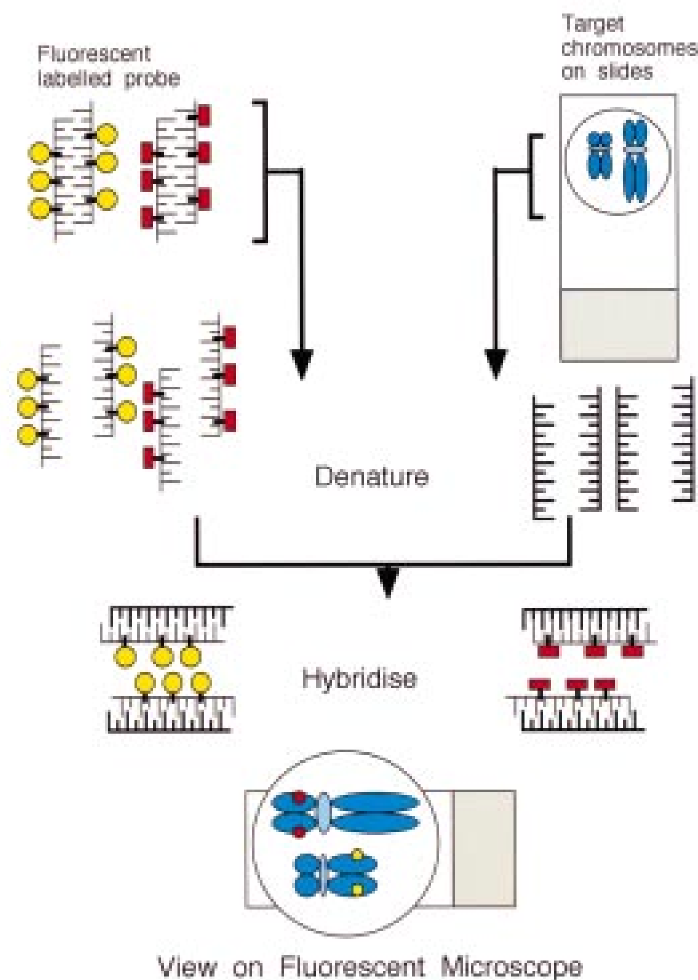

Figure 1 Schematic illustration of the principles of FISH. Two colour FISH for detection of two single locus sequences on metaphase chromosomes is shown.

- probe labelling
- hybridisation conditions
- image detection of the reporter molecule(s).

HYBRIDISATION

DNA is made up of two antiparallel strands of linear arrays of nucleotides (purines and pyrimidines) on a sugar-phosphate backbone, paired thus: adenine (A) with thymidine (T); cytidine (C) with guanidine (G).

The nucleotides on each strand usually pair in a very precise way with the nucleotides on the opposite strand thus: A with T and C with G or vice versa. This phenomenon is known as complementarity. Each pairing of nucleotides in this manner is known as a base pair. The exquisite ability of a sequence of single stranded DNA to recognise its complementary opposite strand by means of the above rules of base pairing is the key to understanding the basis of the technique.

Like any chemical reaction, the rate and fidelity of the pairing of nucleotide molecules depends upon a number of physical parameters that can be manipulated to enhance the specificity of hybridisation.[11–13] Factors that determine the specificity of hybridisation include:

- adequate specimen fixation (frozen samples, paraffin wax embedded sections, etc)
- adequate target denaturation (of double stranded target DNA)
- hybridisation temperature
- hybridisation time
- hybridisation conditions (for example, salt concentration).

A typical FISH detection strategy for metaphase chromosome preparations is shown in fig 1.

Categories of DNA probes for FISH

A powerful and important application of FISH is the detection of a particular gene or genetic sequence (or combination) at the DNA level. There are now a large number of probes available from commercial and other sources.

Probes may be combined in various ways—for example, in chromosome-specific libraries that allow whole chromosomes to be identified or "painted" with the technique. Probes may be delivered in a variety of vectors (for example, plasmids, cosmids, YACs (yeast artificial chromosomes), PACs (P1 filamentous phage artificial chromosomes) or BACs (bacterial artificial chromosomes)). Direct DNA amplification of a DNA sequence using the polymerase chain reaction (PCR), incorporating a suitable reporter molecule, is also widely used.

Various families of probes are available for particular applications as outlined below. Figure 2 illustrates some of these applications.

PROBE FAMILIES

- Whole chromosome paints[10 14–16]
- Chromosome arm specific paints[17]
- Chromosome specific centromeres[18]
- α
- β
- Satellite III
- Chromosome arm specific telomeres[19–22]
- Multicopy, multilocus, sequences
- Pan-telomeric
- Pan-centromeric
- Species specific repeat sequences (for example, Alu)
- Unique or low copy sequences
- Gene sequences
- Non-coding sequences

Complementary target sequences for these probes can be recognised on metaphase chromosomes, interphase nuclei from a variety of fresh or archival sources, including paraffin-wax embedded tissue sections.[23 24]

Clinical applications in cytogenetics

PRINCIPLES

FISH is generally used either to complement classic staining methods or as a substitute for chromosome identification at metaphase or interphase. In particular FISH demonstrates the qualities listed below and illustrated in figs 3–5, for various diagnostic and/or prognostic applications.

Sensitivity

FISH can detect cryptic chromosomal deletions and rearrangements, not detectable by conventional means:

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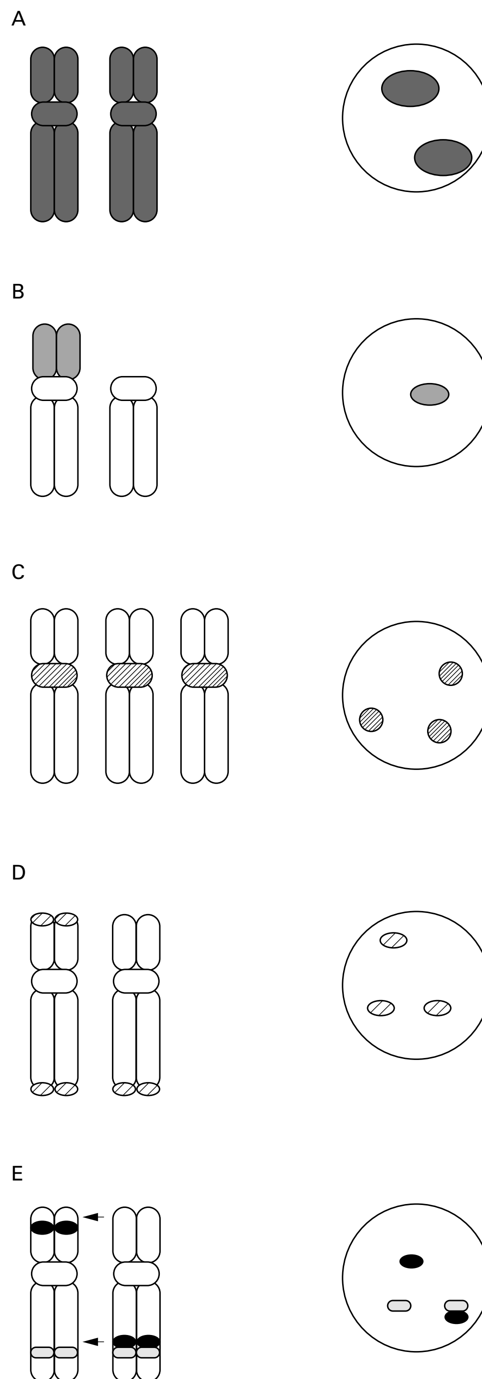

Figure 2 Schematic illustration of the diagnostic application of various classes of probes. (A) Whole chromosome paint; (B) whole chromosome arm paint; (C) single chromosome specific centromere probe; (D) single chromosome arm specific subtelomere probes; (E) single gene probes used in combination. Probe hybridisation illustrated in metaphase chromosomes (left) and interphase nuclei (right). In practice, scoring of interphase nuclei is not used diagnostically for (A), (B), and (D).

- detection of submicroscopic microdeletions at the chromosomal level[25 26] and at the DNA fibre level[27 28] in both haploinsufficiency syndromes (for example, William's syndrome[26]) or single gene disorders (for example, Duchenne's muscular dystrophy[28])
- detection of gene fusion events in malignancy[29]
- detection of hidden aneuploidy.[30]

Specificity

By using a particular probe or probes, chromosomal material of unknown or uncertain origin can be identified:

- identification of marker chromosomes[31]
- identification of chromosomal variants or polymorphisms.[32]

Efficiency

FISH allows rapid screening of a large number of metaphases or interphases for a particular chromosome or other target sequence:

- rapid screening for chromosomal mosaicism[33]
- rapid screening for chromosomal aneuploidy (for example, Down's syndrome) in prenatal samples[34 35]
- monitoring residual disease status in patients with leukaemia[37–39]
- monitoring sex mismatched bone marrow engraftment after transplantation.[40 41]

Discrete information is obtained for each cell, which is an important advantage of the technique. FISH can also be combined with other identification methods such as immunophenotyping of individual cells (see section "Current developments and emerging applications").

Applicability

FISH allows interphase cells to be screened from a wide variety of tissues not directly accessible with conventional cytogenetics.[40 42–44] Some examples are given below:

- human lung carcinoma tissue[45]
- endometrial tissue (aneuploidy in endometriosis)[46]
- uncultured chorionic villus samples[47]
- fetal nucleated erythrocytes identified in maternal blood[48]
- archival autopic heart specimens.[49]

FISH may also be applied to buccal smear samples[50] where venous blood is unavailable for cytogenetic analysis, or to blood smears, where an extremely rapid result is required.[51] The widely applicable technique of touch printing[52] is a valuable method of transferring a single thickness of cells from fresh tissue sections to slides for FISH analysis.

The FISH technique has also provided a great deal of information about chromosome behaviour at meiosis, an area of study hitherto limited by the inability of conventional techniques to identify unequivocally individual chromosomes. FISH allows normal and abnormal chromosomes to be tracked through all stages of meiosis[53 54] Rapid, direct analysis of large numbers of the chromosomal complements of sperm, the products of male meiosis, has been successfully performed using FISH.[55–57]

FISH DETECTION OF mRNA

FISH can be used to detect single stranded messenger RNA in situ.[58–60] This is a potentially important application of the FISH technique because it provides direct visual evidence of gene expression from a particular chromosome. It is technically demanding at present but may have applications in the future in a

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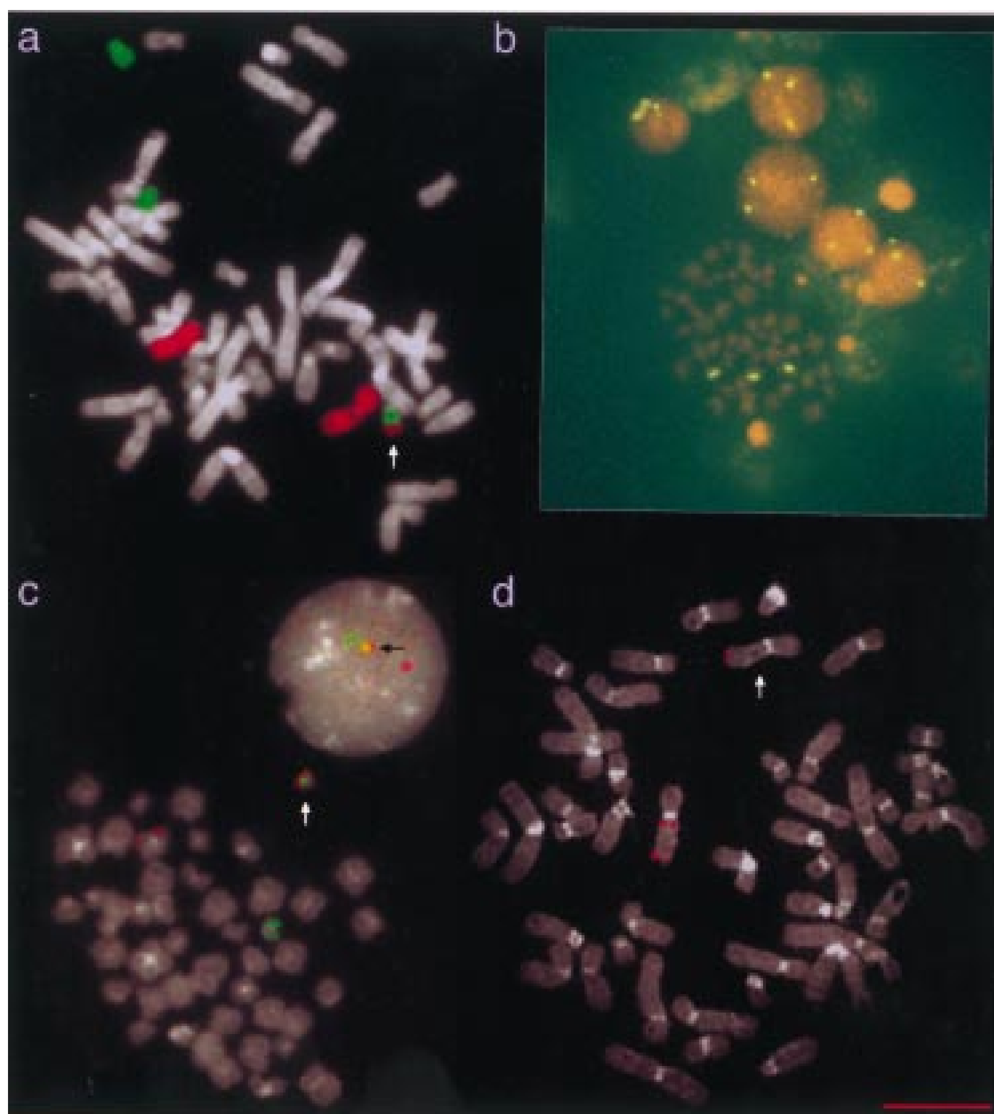

Figure 3 (a) Characterisation of an additional marker chromosome, der(22)t(11;22) (q23.3;q11.2) (arrow); derived from chromosome 11 (red signal) and chromosome 22 (green signal). (b) Detection of trisomy 12 with a chromosome 12 centromere specific probe (yellow signal) in metaphase and interphase cells from a patient with chronic lymphocytic leukaemia. (c) Detection of bcr-abl gene fusion in chronic myeloid leukaemia. bcr probe (green label), abl probe (red label). Metaphase shows bcr-abl fusion on Philadelphia chromosome (white arrow) and an interphase cell (black arrow). (d) Microdeletion in the long arm of chromosome 7 at 7q11.23 identified with FISH (arrow) and seen in William's syndrome. A control probe that maps to the end of the long arm identifies the submicroscopically deleted chromosome 7.

number of clinical disorders. For instance, in Beckwith-Weidemann syndrome[61] the pattern of expression of H19 and IGF2 genes on the short arm of chromosome 11 may provide evidence for uniparental disomy associated with the disorder. In another example the presence of an mRNA product of a particular gene—for example, HbF in fetal erythroblasts,[48] may provide a novel method of identification of fetal cells in maternal venous blood for prenatal diagnosis.

FISH nomenclature

The International System for Human Cytogenetic Nomenclature (ISCN) is an internationally agreed nomenclature for describing all human cytogenetic numerical and structural abnormalities. A standing committee ensures that the nomenclature is kept up to date. The latest edition (ISCN 1995) includes a section on the description of abnormalities confirmed or confirmed by the FISH technique.[62]

FISH refinements

FLUORESCENCE ACTIVATED CHROMOSOME SORTING

In brief, chromosomes stained with two fluorescent dyes, Chromomycin A3 (GC content) and Hoechst 33258 (AT content), can be sorted on a fluorescence activated cell sorter (FACS) according to the relative intensity of fluorescence of the two dyes, which in turn is proportional to chromosome size. Post-sorting amplification techniques[63] allow the sorted chromosome of interest to be flour-labelled and used as defined libraries of probes or paints. The technique was originally exploited to produce paints for each human chromosome, 1–22,X,Y.[10] It has also been used to

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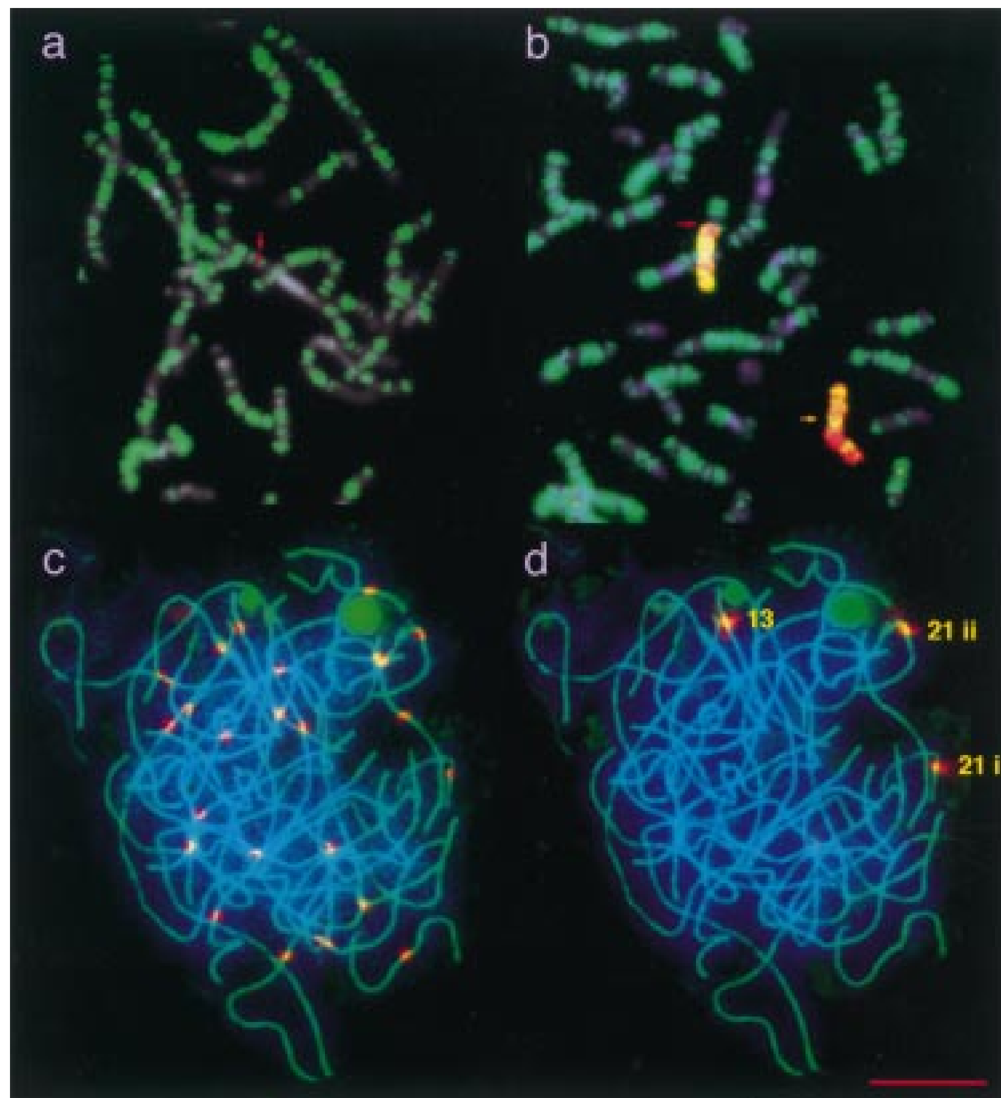

Figure 4 (a) Mapping of the human T cell receptor zeta chain gene (red signal) to chromosome 1 at 1q23.1. Banding with FITC conjugated anti-BrdU monoclonal antibody allows chromosome identification. (b) Whole chromosome X paint (wcpX) allows delineation of the breakpoint in a case involving a translocation between chromosomes X and 6. Der(X)t(X;6) (p11.2;p21.1) chromosome (red arrow); normal chromosome (yellow arrow). (c) Microspread of a pachytene human oocyte from 21 week Down's syndrome fetus (47,XX,+21) labelled with an antisynaptonemal complex (SC) antibody (blue-green) and a CREST antibody that labels centromeres (red). (d) The same spread labelled with anti-SC antibody and an alphoid 13/21 centromere probe (red). This helps to identify a normal 21 bivalent (21ii), a normal 13 bivalent (13), and an additional 21 univalent (21i). The last is consistently visibly thinner than the other bivalents in the nucleus.

characterise abnormal chromosomes, for which the term reverse painting was coined.[64]

MICRODISSECTION

The ability to microdissect DNA from chromosomes in metaphase spreads allows small marker chromosomes to be identified. The microdissected DNA is amplified[63] and then painted back onto normal metaphase spreads so that the chromosomal origin of the microdissected DNA can be identified.[65]

AS A TOOL IN GENE MAPPING

FISH, on metaphase chromosomes, has already proved to be a very powerful gene-mapping tool.[66] Recent refinements exploit the fact that FISH can be applied to interphase nuclei, mechanically stretched chromosomes,[67] or DNA fibres[68] to order DNA sequences, with increasingly greater resolution.

Current developments and emerging applications

Rapid developments are taking place in the automation of image capture, processing, and presentation. All these advances offer the prospect of greater speed and accuracy in answering questions concerning subtle and complex alterations in genome structure and size.

MULTISINGLE OR DUAL COLOUR LABELLING OF DIFFERENT PAIRS OF PROBES ON THE SAME SLIDE

Technology is available that allows metaphase spreads to be screened with a battery of probes on a single slide using either one or two colour fluor-labelling. Specially prepared template slides are divided into a predetermined number of target areas, each of which is primed with a small volume of metaphases.[20 69] Probes in solid-phase are then placed over the metaphases using a multiprobe device and in the

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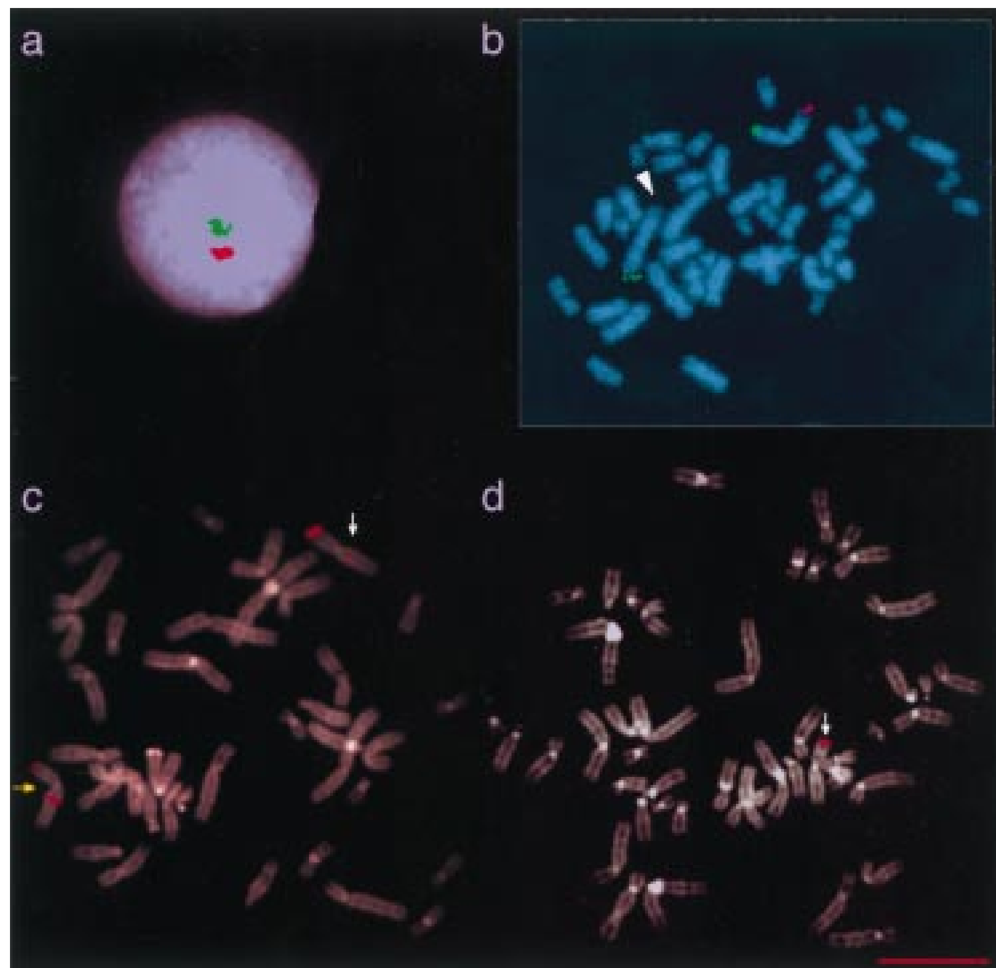

Figure 5 (a) Interphase cell showing X centromere (DXZ1) in red and Y heterochromatin (DYZ3) in green. Rapid screening for sex chromosome status of large numbers of cells is possible with these probes. (b) Multiprobe FISH showing the "1" square, showing a de novo telomeric deletion of the short arm of chromosome 1 (loss of red signal) (arrow). (c) Cross hybridisation of Y euchromatin paint (wcpY) to both X chromosomes (arrows) in an XX male with an Xp/Yp interchange. Cross hybridisation of limited intensity is seen in normal individuals reflecting partial X/Y homology. In this case the interchange results in greater signal intensity in the one X chromosome (upper right). (d) In case (c) the abnormal interchange in confirmed by the presence of a Y short arm specific probe (GMGY10) hybridising to the short arm of one X chromosome only (arrow).

presence of a suitable reagent, the probes themselves are released into solution and hybridisation occurs. This approach has been applied successfully to identifying cryptic subtelomeric deletions and rearrangements in a proportion of patients with idiopathic mental retardation (figs 2 and 5B).[20 22]

SKY-FISH/MULTICOLOUR FISH (M-FISH)

These are two technically related innovations both of which allow simultaneous identification of many different chromosomes, with each chromosome displayed in a characteristic colour.[70–72] Multifluor FISH or M-FISH, uses a small pool of fluorochromes. Each chromosome paint consists of a unique combination of different fluorochromes. These combination paints are then applied to metaphase spreads and the resulting signals collected individually through epifluoresence filter sets, and the images pooled and processed.

Spectral karyotyping or SKY-FISH uses a similar combinatorial labelling approach but the images are collected using a combination of Fourier spectroscopy and CCD imaging. Spectral imaging allows simultaneous measurement of the fluorescence emission spectrum at all sample points.[72–75] A further refinement is the so-called barcode approach that exploits the fact that somatic cell hybrids retain syntenic fragments of human chromosomes on a mouse or Chinese hamster background that can be used as a source of human chromosome fragment specific probes. By selecting a suitable panel of fragments and using multicolour labelling each chromosome acquires a characteristic barcode appearance that can be screened for rearrangements, deletions, etc.[76 77]

COMPARATIVE GENOMIC HYBRIDISATION

Comparative genomic hybridisation (CGH) analysis is a powerful technique to identify the chromosomal location of chromosome gains, losses, or deletion or amplification events. Crucially, it does not require prior preparation of metaphase spreads and is therefore applicable to any cellular biological tissue. A good introduction to the principles of the technique is provided by Buckle and Kearney.[78] Examples of CGH applications for solid tumours, are provided by Kallioniemi *et al*.[79] Briefly, differentially

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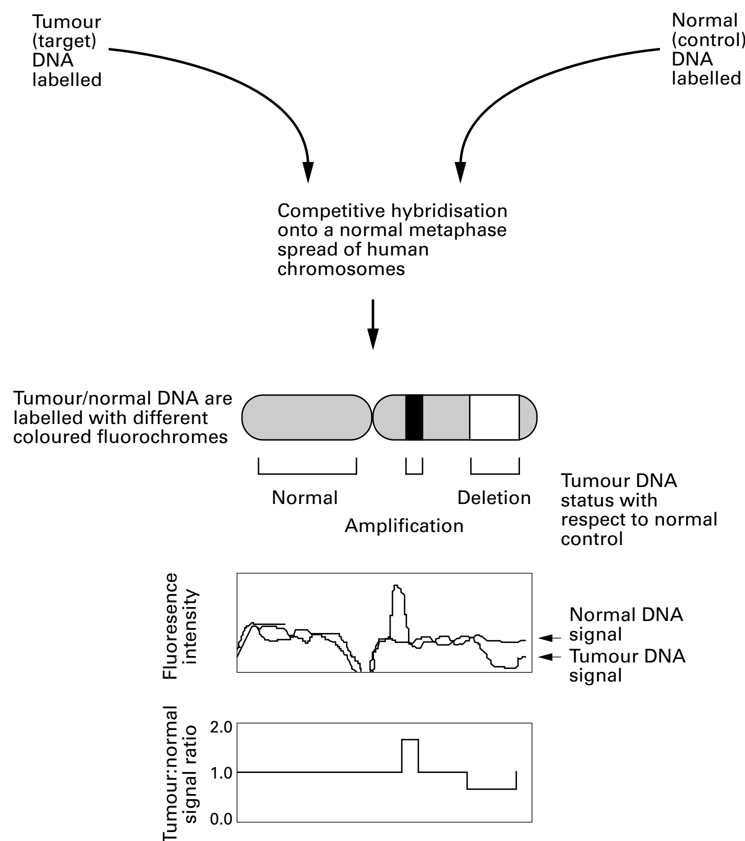

Figure 6 Representation of comparative genomic hybridisation.

fluor-labelled control and target DNA are titrated against each other in a competitive hybridisation reaction onto normal metaphase spreads. Colours generated after hybridisation indicate either a relative excess or deficiency of target DNA (relative to a control) or equality of hybridisation.[80 81] Figure 6 outlines the technique.

The technique has already been widely used in analysis of tumour cell genomes, partly because of the low index and yield of poor quality spreads from solid tumours. Genomic comparison of primary breast carcinomas and their metastases has been performed with this technique.[82] An excellent illustration of the power of the technique is work by Hemminki *et al* who used CGH as a starting point to map a critical gene in Peutz-Jeghers syndrome to the short arm of chromosome 19.[83 84] CGH has also been successfully applied to detect cryptic subtelomeric deletions[85] and additional chromosomal material of unknown origin.[86] The possibility of combining CGH with CHIP based DNA-matrix based technology is also being explored.[87]

FLUORESCENCE IMMUNOPHENOTYPING/ INTERPHASE FISH (FICTION)

A technique that allows karyotypic analysis of morphologically and immunologically classified cells—the so-called MAC (morphology, antibody, chromosomes) technique—was first described by Knuutila *et al*.[86] More recently, immunophenotyping has been successfully combined with FISH in examining mitotic cells in haematological malignancies[88] and in the analysis of meiosis I human spermatocytes.[54] Particularly informative studies in haematological malignancies have made use of combined immunophenotyping and interphase FISH to identify the chromosomal status of subpopulations of cells.[89] This combined approach has been used to identify minimal residual disease[90] or to predict haematological relapse.[39] The acronym FICTION (fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms) has been coined to describe this approach.[91]

Conclusion

This review has attempted to outline the principles of FISH and to convey the scope of the technique. A number of the ways in which the technique is being developed has been highlighted. The wide variety of applications that are possible in many different areas of pathology have been summarised.

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