

## Review

# Applications of fluorescence *in situ* hybridization (FISH) in detecting genetic aberrations of medical significance

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Fluorescence *in situ* hybridization (FISH) is a powerful technique used in the detection of chromosomal abnormalities. The high sensitivity and specificity of FISH and the speed with which the assays can be performed have made FISH a pivotal cytogenetic technique that has provided significant advances in both the research and diagnosis of haematological malignancies and solid tumours. From a medical perspective, FISH can be applied to detect genetic abnormalities such as characteristic gene fusions, aneuploidy, loss of a chromosomal region or a whole chromosome or to monitor the progression of an aberration serving as a technique that can help in both the diagnosis of a genetic disease or suggesting prognostic outcomes. FISH can also be applied to such research applications as gene mapping or the identification of novel oncogenes or genetic aberrations that contribute towards various cancers. FISH is based on DNA probes annealing to specific target sequence of sample DNA. Attached to the probes are fluorescent reporter molecules which under fluorescence microscopy confirm the presence or absence of a particular genetic aberration when viewed under fluorescence microscopy. The technique has recently evolved to allow screening of the whole genome simultaneously through multicolour whole-chromosome probe techniques such as multiplex FISH or spectral karyotyping, or through an array-based method using comparative genomic hybridization. This simple, yet effective, technique has revolutionized cytogenetics and has become well established in its potential as a diagnostic and discovery tool in the fight against cancer.

**Key words:** FISH, fluorescence microscopy, chromosomal aberrations.

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## Introduction

Refinements in cytogenetic techniques over the past 30 years have allowed the increasingly sensitive detection of chromosome abnormalities in haematological malignancies, with the advent of fluorescence *in situ* hybridization (FISH) techniques providing significant advances in both diagnosis and research of haematological malignancies and solid tumours<sup>1</sup>.

Chromosome banding techniques (Giesma staining) revolutionized cytogenetic analysis and have been pivotal in the understanding of genetic changes in both constitutional and acquired diseases (in particular, the knowledge of the contribution of specific chromosome abnormalities to leukaemia). However, the resolution of banding analysis is

such that it can only detect rearrangements that involve >3 Mb of DNA<sup>1</sup>. Banding techniques are limited to mitotically active cells with the additional problem of the difficulties involved in deciphering highly rearranged chromosomes using a monochrome banding pattern. The introduction of FISH in the late 1980s, as a technique that can readily detect trisomies and translocations in metaphase spreads and interphase nuclei using entire chromosome-specific DNA libraries, was heralded as a further revolution in cytogenetic analysis<sup>1, 2</sup>. The high sensitivity and specificity of FISH and the speed with which the assays can be performed have made FISH a powerful technique with numerous applications, and it has gained general acceptance as a clinical laboratory tool<sup>3</sup>.

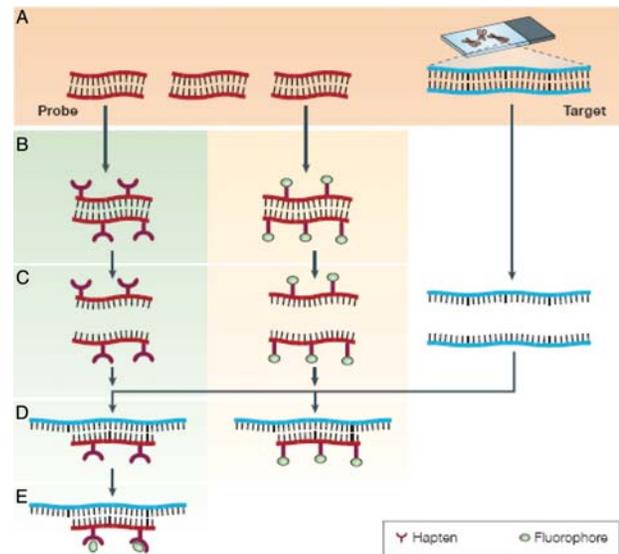
‘Chromosome painting’, competitive hybridization using entire chromosome-specific libraries for chromosomes as probes and human genomic DNA as the competitor,<sup>2</sup> was one of the first applications of FISH. It provided intense and specific fluorescent staining of human chromosomes in metaphase spreads and interphase nuclei, allowing the distinctive identification of chromosomes involved in complex rearrangements. The advent of the Human Genome Project has made available a repertoire of single-locus probes that have provided a significant boost to gene mapping strategies and led to the identification of the breakpoints of consistent translocations.<sup>4, 5</sup> The first specific translocation identified in human neoplasia was t(9;22)(q34;q11) resulting in the Philadelphia chromosome<sup>6, 7</sup>; and the delineation of critical deleted regions associated with specific disease subtypes.<sup>1, 8</sup>

FISH is essentially based upon the same principle as a Southern blot analysis, a cytogenetic equivalent that exploits the ability of single-stranded DNA to anneal to complementary DNA. In the case of FISH, the target is the nuclear DNA of either interphase cells or of metaphase chromosomes affixed to a microscope slide, although FISH can also be performed using bone marrow or peripheral blood smears, or fixed and sectioned tissue<sup>3</sup>. Once fixed to a microscope slide, the desired cells are hybridized to a nucleic acid probe. This anneals to its complementary sequence in the specimen DNA and is labelled with a reporter molecule which is either an attached fluorochrome, enabling direct detection of the probe via a coloured signal at the hybridization site visualized by fluorescence microscopy, or a hapten that can be detected indirectly.<sup>3, 9</sup> This second method relies on immunohistochemistry (IHC) for probe detection which is based on the binding of antibodies to specific antigens, once antigen-antibody binding occurs, it is demonstrated with a coloured histochemical reaction visible by light microscopy or fluorochromes with ultraviolet light.<sup>10</sup> IHC is limited by the availability of antibodies. For direct detection FITC, Rhodamine, Texas Red, Cy2, Cy3, Cy5 and AMCA are the most frequently used reporter molecules. Biotin, Digoxigenin and Dinitrophenol are the reporter molecules typically used for indirect detection methods such as IHC.<sup>9</sup> Fig. 1 gives a diagrammatic presentation of an overview of the FISH process.

## Genetic aberrations

Chromosomes consist of DNA, histones, and non-histone proteins and are organized during interphase into domains. In most cells, chromosomes are visible via microscopy only during the process of cell division. G<sub>0</sub>/G<sub>1</sub> chromosomes contain one DNA molecule which when replicated in the S phase of the mitotic cell cycle yields two sister chromatids that are both replicas of the original DNA molecule.<sup>11</sup>

During the cell cycle, ubiquitous enzymes known as topoisomerases readily mediate DNA double-strand breaks to alter the superhelical state in DNA. This temporary cleavage



**Figure 1.** The principles of fluorescence *in situ* hybridization. (A) The basic elements are a DNA probe and a target sequence. (B) Before hybridization, the DNA probe is labelled indirectly with a hapten (left panel) or directly labelled via the incorporation of a fluorophore (right panel). (C) The labelled probe and the target DNA are denatured to yield single-stranded DNA. (D) They are then combined, which allows the annealing of complementary DNA sequences. (E) If the probe has been labelled indirectly, an extra step is required for visualization of the non-fluorescent hapten that uses an enzymatic or immunological detection system. Finally, the signals are evaluated by fluorescence microscopy. Adapted from Speicher and Carter.<sup>35</sup> Reprinted by permission from Macmillan Publishers Ltd: *Nat Rev Genet* 6: 782–792, copyright 2005.

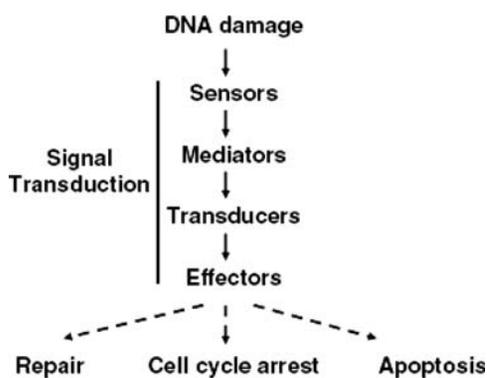
is essential for topoisomerases to carry out their primary cellular functions, including their roles in DNA replication, transcription, chromosome condensation and segregation, and the maintenance of genome stability. However, the action of these enzymes can promote illegitimate recombination that may lead to chromosomal aberrations.<sup>12</sup>

Although the DNA replication process is remarkably accurate, human cells are estimated to contain approximately 10 double-strand breaks per cell cycle, as estimated by the incidence of spontaneous sister chromatid exchanges. If left unrepaired, such damage results in the loss of chromosomes and/or the induction of cell death. If imprecisely repaired, the damage leads to mutations and chromosomal rearrangements. These DNA double-strand breaks (which may result in gene malfunction) are considered to be critical primary lesions in the formation of chromosomal aberrations and can occur in both somatic and germ-line cells.<sup>11, 13</sup> Fortunately, cells devote significant resources to the repair of DNA double-strand breaks, inducing several cellular responses including DNA repair via gene conversion and recombination-dependent DNA replication; cell cycle checkpoint activities associated with biochemical pathways resulting in the delay or arrest of cell cycle progression; and the triggering of apoptotic pathways.<sup>13, 14</sup>

The mitotic, or spindle assembly, checkpoint is a mechanism that arrests the progression to anaphase to ensure accurate chromosome segregation. Mitotic arrest is induced when errors occur in the spindle structure or in the chromosomal alignment on the spindle. Loss of mitotic checkpoint control is a common event in human cancer cells, which is thought to be responsible for chromosome instability with various defects causally implicated in tumourgenesis. However, how mitotic arrest contributes is not well defined. Cells with a defective mitotic checkpoint are more resistant to several types of anticancer drugs from microtubule disruptors to DNA damaging agents. In addition, inactivation of key mitotic checkpoint proteins such as BUB (budding uninhibited by benzimidazole) and MAD (mitotic arrest deficient) are influential in drug resistance in mitotic checkpoint defective cancer cells.<sup>15, 16</sup>

The p53 tumour suppressor protein plays a central role in the decision of a cell to undergo either cell-cycle arrest during the G<sub>1</sub> phase, or apoptosis, to protect the genome from potentially mutagenic damage from diverse stresses, including DNA damage, hypoxia and the activation of oncogenes. It has been estimated that about 50% of all tumours have mutations in p53, and the p53 pathway may be nonfunctional for other reasons in many more. p53 is also known for its role in monitoring genomic stability, but the mechanisms underlying this function are not fully understood. Nevertheless, it is known that genetic insults activate p53, which in turn induce downstream repair genes including *GADD45*, *p48XPE* and *XPC* that are involved in the nucleotide excision repair and base excision repair processes.<sup>14, 17, 18</sup> Cellular responses to DNA damage are outlined in Fig. 2.

Mutations can occur in the genomes of all dividing cells as a result of misincorporation during DNA replication or

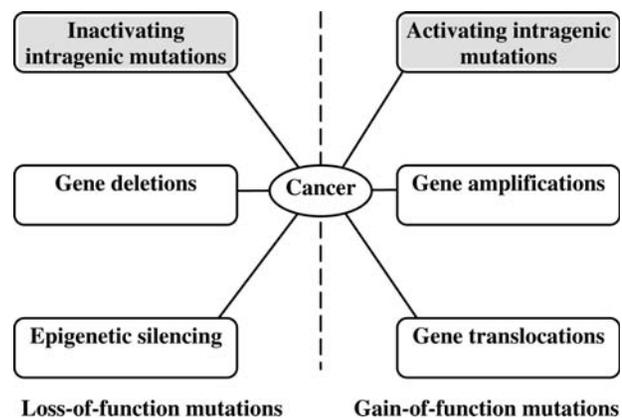


**Figure 2.** Theoretical association of the signal transduction of checkpoint responses. DNA damages are recognized by sensor proteins. The signals are transmitted to transducers (mainly kinases) via mediators and the regulated transducer molecules suppress effector kinases, such as Cdk5 and Cdc7, thereby arresting the cell cycle at the specific phases. Adapted from Niida and Nakanishi by permission of the UK Environmental Mutagen Society.<sup>14</sup>

through exposure to exogenous mutagens such as ionizing radiation or endogenous mutagens<sup>19</sup> (Fig. 3). Cancers result from clonal proliferations that arise from an accumulation of mutations and other heritable changes that confer selective growth advantages in susceptible cells. A central aim of cancer research has been to identify the mutated genes that are causally implicated in oncogenesis. So far, abnormalities in about 350 genes (more than 1% of our genome) have been implicated in human cancers, but the true number is unknown. This illustrates striking features in the types of sequence alteration and protein domains that are encoded in the cancer classes in which oncogenic mutations have been identified.<sup>19–21</sup>

Cancer genomes carry two biological classes of somatic mutation arising from these various processes. ‘Driver’ mutations emerge as a result of selective pressure during tumourgenesis. They confer growth advantage on the cell in which they occur, are directly implicated in cancer development and therefore are positively selected (passed on to the cancer progeny). Conversely, ‘passenger’ mutations are incidental, possible products of genome instability or the large number of cell divisions that lead from a single transformed cell to a clinically detectable cancer. They are present in the cell that was the progenitor of the final clonal expansion of the cancer, are biologically neutral and do not confer growth advantage.<sup>19, 21</sup>

While certain types of chromosomal aberrations are lethal, others may lead to oncogenic transformation by



**Figure 3.** The diversity of mutations that can initiate human cancer. Intragenic mutations—highlighted—are small nucleotide changes that may be inherited by the cancer progeny from the progenitor cell, resulting in either a loss-of-function of genes or a gain-of-function of genes. Epigenetic silencing involves heritable modifications of nucleotides and histones in regulatory regions of genes, leading to suppression of gene expression in the absence of DNA mutations. Translocations lead to the fusion of DNA fragments from different chromosomal regions, either creating an abnormal fusion protein or leading to aberrant expression of a normal gene. These so-called ‘driver’ mutations contribute towards oncogenesis. Adapted from Niida and Nakanishi by permission of the UK Environmental Mutagen Society.<sup>14</sup>

inactivation of a tumour suppressor gene or activation of an oncogene via generating novel fusion proteins capable of initiating carcinogenesis. Increased frequencies of chromosomal aberrations are correlated with elevated risks of cancer and certain human neoplasias are associated with defined chromosomal aberrations branding chromosomal aberrations a hallmark of all tumour cells.<sup>11</sup>

The molecular mechanisms for chromosomal aberrations are not yet fully understood. There are two classical, and one modern, theory that attempt to explain the generation of chromosomal aberrations.<sup>11</sup> The 'breakage and reunion theory'<sup>22, 23</sup> proposes that breaks in the chromosome axis may be rejoined to the original structure; lead to exchange-type aberrations by rejoining of different breaks or no rejoining resulting in DNA breaks.<sup>11, 24</sup> The 'exchange theory'<sup>25</sup> assumes the presence of unstable lesions that, when coming into close contact, initiate an exchange mechanism resulting in exchange-type aberrations or DNA breaks.<sup>11, 24</sup> The 'molecular theory'<sup>26</sup> suggests that exchange-type aberrations result from an induced DNA double-strand break, as a result of the recombinational repair mechanism, leading to the production of a second enzymatically induced DNA double-strand break at the site of recombination, resulting in a heteroduplex formation.<sup>11, 24</sup> Staining of chromosomes with Giesma allows analysis of different types of chromosomal aberrations such as polycentric chromosomes, ring chromosomes, chromatid interchanges and fragments. Other chromosomal aberration types such as reciprocal translocations and inversions are not normally recognizable with Giesma staining but can be visualized by FISH.<sup>11</sup>

## Probes

The potential of almost all applications of *in situ* hybridization is greatly enhanced by multicolour detection of simultaneously hybridized probes. This is particularly useful when structural chromosome aberrations involving different chromosomal regions are to be diagnosed, or when several numerical aberrations should be detected in parallel.<sup>9</sup> One of the most important considerations in FISH analysis is the choice of probe. A wide range of probes can be used, from whole genomes to small cloned probes (1–10 kb). There are broadly three types of probe, each with a different range of applications: whole-chromosome painting probes; repetitive sequence probes and locus-specific probes.<sup>1</sup>

Chromosome 'painting' refers to the hybridization of fluorescently labelled chromosome-specific, composite probe pools to cytological preparations, which allows the visualization of individual chromosomes in metaphase or interphase cells and the identification of both numerical and structural chromosomal aberrations in human pathology with high sensitivity and specificity.<sup>27</sup> The whole-chromosome painting probes are complex DNA probes derived from a single type of chromosome that has usually

been flow-sorted (or microdissected), amplified and labelled by degenerate oligonucleotide polymerase chain reaction to generate a 'paint' which highlights the entire chromosome homogeneously along its length (through this method chromosome arm-specific paints and region-specific paints can also be generated).<sup>1</sup> This type of probe is most useful for clarifying cytogenetically visible structural or numerical chromosome rearrangements in metaphase, but painting probes are not helpful in the analysis of interphase cells because the signal domains are so large and diffuse.<sup>1, 3</sup> An example of the power of FISH is that of the genetic translocation t(12;21)(p12;q22) in acute lymphoblastic leukaemia (ALL), which was first detected through the use of FISH with chromosome painting probes.<sup>3</sup> The widespread use of chromosome painting in nonspecialized laboratories has also become possible due to improved microscope hardware (microscopes and optical filters), the use of sensitive digital imaging devices (CCD cameras, confocal laser scanning microscopes) and an increasing number of suitable DNA haptenization and fluorescent labelling systems.<sup>27</sup> Whole chromosome painting is available for every human chromosome, allowing the simultaneous painting of the entire genetic complement in 24 colours. This promptly led to the development of two independent FISH techniques, multicolour FISH (M-FISH) and spectral karyotyping (SKY) that have both been invaluable in diagnostic and research applications (discussed later).

Repetitive sequence probes hybridize to specific chromosomal regions or structures that contain short sequences which are present in many thousands of copies.<sup>1, 3</sup> Examples of this probe type are Pan-telomeric probes targeting the tandemly repeated (TTAGGG) sequences present on all human chromosome ends or centromeric probes that target the  $\alpha$  and  $\beta$  satellite sequences, which flank the centromeres of human chromosomes.<sup>1, 3</sup> In most instances, these sequences are distinct, such that an  $\alpha$ -satellite probe derived from one chromosome will hybridize to that chromosome only,<sup>3</sup> however pan-centromeric probes, which target all human centromeres, are also available.<sup>1</sup> Satellite DNA probes hybridize to multiple copies of the repeat unit present at the centromeres resulting in a two very bright fluorescent signals in both metaphase and interphase diploid cells, hence making centromere-specific probes particularly suitable for the detection of monosomy, trisomy and other aneuploidies in both leukaemias and solid tumours.<sup>3</sup> A centromere-specific probe for chromosome 8 has been used to detect trisomy by FISH in newly diagnosed patients with a myelodysplastic syndrome or acute myeloid leukaemia (AML), and to monitor the response to therapy.<sup>3</sup>

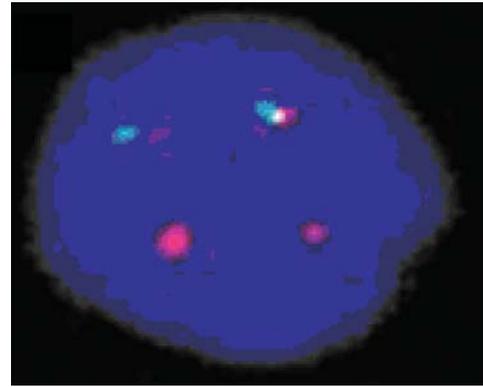
The third type of probe, locus-specific probes, are usually genomic clones, which vary in size depending on the nature of the cloning vector, from plasmids (1–10 kb) to the larger PAC, YAC and BAC vectors (80 kb to 1 Mb). Probes of this classification are particularly useful for detecting structural

rearrangements such as specific chromosomal translocations, inversions or deletions in both metaphase and interphase.<sup>1,3</sup> Using multicolour FISH, recurring translocations can be identified in cells by means of genomic probes that are derived from the breakpoints. For example, a locus-specific probe for the *BCR* (breakpoint cluster region) gene at 22q11.2 detected with a green fluorochrome and a locus-specific probe for the *ABL* (Abelson oncogene) gene at 9q34 detected with a red fluorochrome will appear as a bright yellow spot (the combination of green and red fluorochromes) in leukaemia cells when viewed via fluorescence microscopy, characterized by the *BCR/ABL* fusion gene, resulting from  $t(9;22)(q34;q11.2)$ .<sup>3, 28</sup> Interphase FISH can be used for the detection of any chromosome abnormality for which there is an appropriate probe. However, limitations of the technique include an inherently high false-positive rate due to the apparent co-localization of two signals when viewing a three-dimensional nucleus in two dimensions.<sup>1</sup>

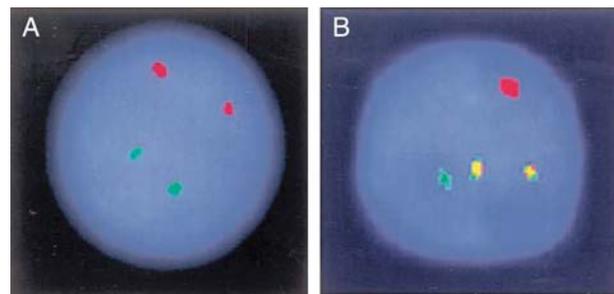
Chase *et al.*<sup>29</sup> emphasized how false positives (and false negatives) could be viewed if the incorrect choice of locus-specific probe was chosen to screen for a translocation. Using the *BCR/ABL* gene fusion as their model, which gives rise to the unique molecular characteristic of chronic myeloid leukaemia (CML), the Philadelphia chromosome,<sup>29, 30</sup> they demonstrated that incorrect assumptions could be made on the presence or absence of this aberration using dual-colour probes. As well as the conventional single fusion signal probe, there are broadly three types of commercially available dual-colour probes: translocation probes, 'split-apart' rearrangement probes and probes for deletion detection.<sup>1, 3</sup> As discussed above, the single-fusion signal probe for *BCR/ABL* contains the 5' portion of the *BCR* gene exhibiting green fluorescence and the 3' portion of the *ABL* gene demonstrating red fluorescence; with a yellow fusion signal observed on the Philadelphia chromosome.<sup>3</sup> However, probes of this type have a relatively high number of false-positive fusion signals (2–6%) as a result of the close proximity and combination of target chromosomes in interphase nuclei, limiting their use to metaphase cells which are typically more uncommon when scoring for mutations.<sup>3, 28</sup> The next generation of *BCR/ABL* probes rectified this problem. Extra-signal probes work on the same principle as their predecessor but part of the DNA sequences recognized by one of the probes (*ABL*) remains at the original site, giving rise to an extra red signal. Therefore, false positives can be distinguished from genuine fusion signals by the absence of the extra red signal for the 5' *ABL* sequences resulting in improved sensitivity (Fig. 4).<sup>3</sup>

In a dual-fusion probe, the probes for each of the genes involved in the translocation span the breakpoint, resulting in two fusion signals, corresponding to the two derivative chromosomes (Fig. 5).<sup>1, 31</sup>

Dual-colour split-apart rearrangement probes are essentially the reverse of the aforementioned probes. They consist of sequences flanking the gene disrupted by the



**Figure 4.** Interphase FISH on a nucleus using an Extra-signal probe to detect the *BCR/ABL* translocation. The green signal indicates the presence of the *BCR* gene, red signals indicate the presence of the *ABL* gene and the red-green fusion (yellow) signal confirms a *BCR/ABL* translocation. The extra red signal confirms this is not a false positive result. Adapted from Primo *et al.*<sup>34</sup> Reprinted by permission from Macmillan Publishers Ltd: *Leukemia* 17: 1124–1129, copyright 2003.

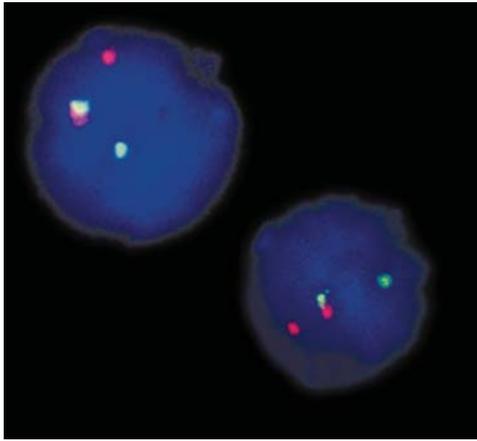


**Figure 5.** Interphase FISH using dual fusion probes on two separate nuclei to detect. (A) Normal nuclei and (B) nuclei containing *BCR/ABL* double fusions indicated by green-red fusion (yellow) signals. Adapted from Dewald *et al* by permission of the American Society of Hematology.<sup>31</sup>

rearrangement. In the germline configuration, a yellow fusion signal is observed, whereas separate red and green signals are observed when the sequences are separated as a result of a translocation. The sensitivity of this probe is exceedingly high with excellent specificity (Fig. 6).<sup>1, 3</sup>

## Multiplex FISH

One of the most appealing aspects of FISH technology is the ability to identify several regions or genes simultaneously, using different colours.<sup>32</sup> In 1996, two groups independently reported a successful 24-colour karyotyping, termed multiplex-FISH (M-FISH)<sup>33</sup> and spectral karyotyping (SKY),<sup>34</sup> respectively.<sup>32</sup> M-FISH (and SKY) allow painting of the entire chromosome complement in a single hybridization through labelling each chromosome with a different combination of fluorophores. Images are collected with a fluorescence microscope that has filter sets for each



**Figure 6.** Interphase FISH on bone marrow nuclei containing the translocation  $t(11;19)(q23;p13)$  using a dual-colour split-apart probe. Green-red fusion (yellow) signals indicate a normal cell. Separate green and red signals indicate the presence of translocations. Adapted from Kearney with permission from Elsevier.<sup>1</sup>

fluorochrome, and a combinatorial labelling algorithm allows separation and identification of all chromosomes, which are visualized in characteristic pseudo-colours.<sup>35</sup> M-FISH and SKY differ only in the method used to discriminate the differentially labelled probes. SKY uses a dedicated imaging system that incorporates a cooled chargecouple device (CCD camera) and Fourier transform spectrometry to analyse the spectral signature at each pixel of the image. M-FISH uses specific narrow bandpass fluorescence filter sets to reduce crosstalk and digital imaging equipment as part of a conventional epifluorescence microscope, with appropriate computer software. The high efficiency of modern epifluorescence microscopes reduce typical exposure times limiting the effects of photobleaching.<sup>32</sup> The achievement of 24-colour FISH-based karyotyping (M-FISH, SKY) has been one of the great successes of molecular cytogenetics in the past decade. The main applications for M-FISH have been the characterization of unbalanced translocations, complex chromosomal rearrangements and marker chromosomes in solid tumours, which are often distinguished by complex karyotypes.<sup>32, 36</sup> In common with other whole-chromosome painting methods, both M-FISH and SKY are not suitable for discriminating intrachromosomal rearrangements such as duplications, deletions or inversions; and the sensitivity and specificity of 24-colour karyotyping depends critically on the fluorochrome combination in the chromosomes involved in rearrangements.<sup>1, 32, 37</sup>

## Comparative genomic hybridization

The preparation of high-quality metaphase spreads, especially from solid tumours, is often difficult. As a consequence, leukaemia, from which metaphase chromosomes

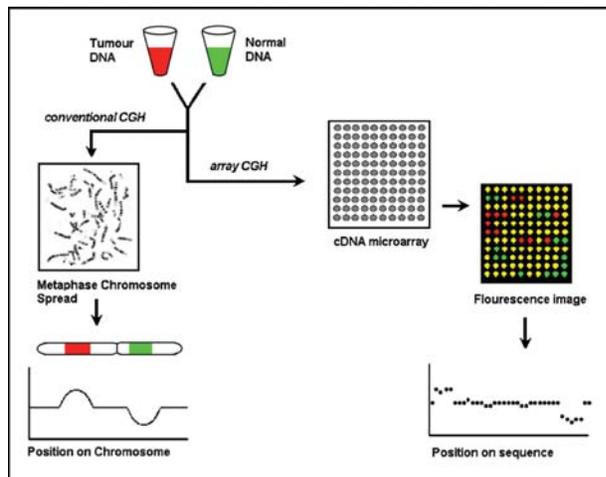
are readily obtained, has been more thoroughly investigated than solid tumours.<sup>35</sup> One of the most significant developments for FISH in relation to genome-wide screening was the introduction of comparative genomic hybridization (CGH) in 1992. This modification of quantitative two colour fluorescence *in situ* hybridization utilizes genomic DNA (overcoming the need for metaphases in array CGH) from the sample under test to generate a map of DNA copy number changes in tumour genomes making it an ideal tool for analyzing chromosomal imbalances in archived tumour material and for examining possible correlations between findings and tumour phenotypes.<sup>1, 38</sup> In CGH, the genomic DNA from the specimen and control DNA extracted from an individual with a normal karyotype (46,XX or 46,XY) are differentially labelled with green and red fluorochromes respectively, mixed in equal amounts and co-hybridized to reference human metaphase chromosomes.<sup>1, 38</sup> The relative difference in DNA content between the normal and specimen DNA is represented by a difference in the green:red fluorescence ratios. For example, if chromosomal material is present in identical copy numbers in both the reference and the specimen genome, the observed fluorescence is a blend of an equal contribution of red and green fluorescence. If chromosomes are lost or chromosomal sub-regions are deleted in the specimen genome, the resulting colour is shifted to red. A gain in certain chromosome in the specimen, such as the amplification of oncogenes, is reflected by a more intense green staining of the respective chromosome in the reference metaphase preparation. The ratios of test to reference fluorescence along the chromosomes are quantified using digital image analysis.<sup>1, 35, 38</sup>

One of the main advantages of CGH is its use as a discovery tool, as it requires no prior knowledge of the chromosome imbalance that is involved.<sup>35</sup> CGH has also contributed significantly to the analysis of haematological malignancies in the identification of (previously unrecognized) high-level amplifications, particularly in chronic lymphocytic leukaemia and non-Hodgkin lymphoma, and as an aid to classification schemes for the lymphomas.<sup>1</sup> The efficiency of the technique allowed it to surpass the number of cases analysed by means of chromosome banding analyses in some cancers.<sup>38</sup> For rearrangements that do not involve genomic imbalances, such as balanced chromosome translocations and inversions, the use of CGH is limited. In addition, whole-genome copy number changes (ploidy changes) cannot be detected. Furthermore, CGH provides no information about the structural arrangements of chromosome segments that are involved in gains and losses.<sup>35</sup>

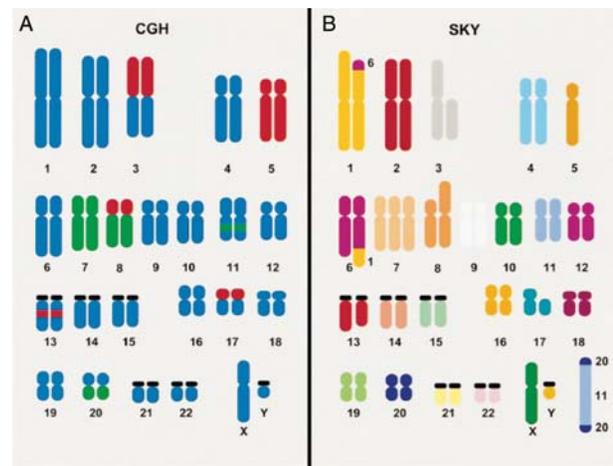
In array CGH, metaphase chromosomes are replaced as the target by large numbers of mapped clones that are spotted onto a standard glass slide greatly increasing the resolution of screening for genomic copy number gains and

losses. In array CGH, the test and normal reference genomes, which are used as probes, are differentially labelled and co-hybridized to a microarray before being imaged.<sup>35</sup> The relative fluorescence intensities are calculated for each mapped clone, with the resulting intensity ratio reflecting the DNA copy number difference (Fig. 7). The resolution of the analysis is restricted only by clone size and by the density of clones on the array. A further advantage is the ease with which array CGH can be automated for high-throughput applications.<sup>35</sup> The flexibility of array design has also allowed the development of specialized arrays for applications such as telomere screening or for specific diseases (for example, B-cell leukaemia), but array CGH is of little use for studying chromosomal abnormalities that do not involve copy number changes, such as inversions or balanced translocations.<sup>35</sup>

Despite these limitations, array CGH has become one of the most widely used cytogenetic techniques in both basic research and molecular diagnostics. It has also altered our view of cancer biology, revealing that tumours of the same type have similar patterns of DNA gains and losses, and that the frequency of changes increases with tumour progression.<sup>35</sup> M-FISH and SKY have emerged as perfect partners for array CGH technologies, providing a powerful approach to gene discovery (Fig. 8). The strength of



**Figure 7.** Comparative genomic hybridization. Genomic DNA is isolated from both the tumour sample and the normal reference sample, labelled with different fluorochromes and mixed in the presence of excess Cot-1 DNA to prevent binding of repetitive sequences. In conventional chromosomal CGH, these are hybridized to normal metaphase chromosomes and the ratio of fluorescence intensities along each chromosome is analysed. Increased DNA copy number (amplification) in the tumour sample will be detected by increased red fluorescence, whereas decreased copy number in the tumour sample will allow more binding of the normal DNA and increased green fluorescence. On the right, a similar hybridization to a cDNA array permits measurement of copy number at a higher resolution. The red and green spots on the fluorescence image represent increased and decreased copy number changes, respectively. Adapted from Manning *et al.* with permission from Elsevier.<sup>42</sup>



**Figure 8.** Schematic presentation of CGH (A) and SKY (B). Both techniques have the distinct advantage that the entire test genome can be analysed in a single experiment (taken from Ried *et al.*<sup>38</sup>). In CGH the simultaneous hybridization of differentially labelled tumour DNA (*green fluorescence*) and reference DNA (*red fluorescence*) to normal metaphase chromosomes allows identification and determination of the chromosomal mapping position of DNA copy number changes in tumour genomes. Regions not affected by copy number changes are shown in blue. A red fluorescence indicates chromosome loss or deletion; whereas a green fluorescence reflects a gain of DNA sequences in the tumour specimen. SKY (or M-FISH) allows simultaneous visualization of all human chromosomes in different colours. The cartoon presents metaphase chromosomes from the same hypothetical tumour genome as in (A). For instance, the trisomy 7 corresponds to the gain detected by CGH. Balanced chromosomal aberrations, such as a reciprocal translocation between chromosomes 1 and 6, do not affect the copy number; therefore, they are not visible by CGH. Adapted from Ried *et al.* with kind permission from Springer Science + Business Media.<sup>38</sup>

M-FISH (or SKY) is in defining translocations and marker chromosomes in complex karyotypes, whereas array CGH can reveal hidden deletions and amplifications.<sup>32</sup>

### Why FISH?

FISH has greatly expanded the capabilities of cytogenetics and pathology laboratories through its high sensitivity, specificity and rapid turnover with a high efficiency of hybridization and detection. Material for FISH can be processed in 4–24 h, and the analysis of 1000–2000 cells accomplished in 15–45 min, enabling the information on the cytogenetic pattern of tumour cells to be achieved within a sufficient time frame for use in treatment strategies.<sup>3, 39</sup> The broad applications of FISH techniques are summarized in Table 1.

A particular advantage of FISH techniques is the possibility to also study chromosomal aberrations in nondividing cells, which is useful for the visualization of chromosomal aberrations directly in cytological preparations and tissue sections. This has led to chromosomal mapping of commonly deleted or amplified regions, providing starting points for the search

**Table 1.** Examples of FISH applications

Diagnostic	Research
The identification of specific chromosome abnormalities	The identification of new non-random abnormalities (by M-FISH or SKY)
The characterization of marker chromosomes	Gene mapping
Interphase FISH for specific abnormalities in cases of failed cytogenetics	Identification of regions of amplification or deletion by CGH
Monitoring disease progression	The identification of translocation breakpoints
Monitoring the success of bone marrow transplantation	The study of 3D chromosome organization in interphase nuclei

Adapted from Kearney with permission from Elsevier.<sup>1</sup>

for a gene (or genes) involved in growth control in certain tumour types.<sup>38</sup> It is imperative to identify genes actively involved in cancer development, because they may provide targets for a directed therapy; however, the issue is complicated by the fact that human populations are genetically heterogeneous, and histologically similar cancers may have resulted from different sets of genetic events. Amplification of oncogenes is an important factor in carcinogenesis and is often correlated to progressive tumour growth and poor prognosis; therefore, identification and characterization of genes subject to amplification can provide valuable molecular tools for evaluation of prognosis in human cancers.<sup>40</sup> The fusion gene *TEL/AML-1* formed by the cryptic t(12;21) detected by FISH is probably the most common genetic defect in childhood. Certain primary AMLs might have a good prognosis, because they are simple diseases with relatively few genetic abnormalities. Their use in combination with other known prognostic indicators permits a more precise molecular delineation of prognostic groups in the development of future clinical childhood ALL trials.<sup>39, 41</sup>

M-FISH (and other multicolour FISH technologies) excel at the characterization of unbalanced translocations, complex chromosomal rearrangements and marker chromosomes. In addition, whole-chromosome painting provided the first evidence that chromosomes occupy discrete 'territories' (or domains) within the interphase nucleus.<sup>1, 32</sup> The application of CGH to identify chromosomal aberrations in human leukaemias, lymphomas and solid tumours added significantly to the understanding of nonrandom, tumour and tumour stage-specific genetic changes, improving diagnostics and diagnostic staging to help devise more carefully adapted therapeutic regimens.<sup>38</sup> CGH can be applied to the analysis of single cells, and such approaches have been used for prenatal diagnosis and analyses of minimal residual disease.<sup>35</sup>

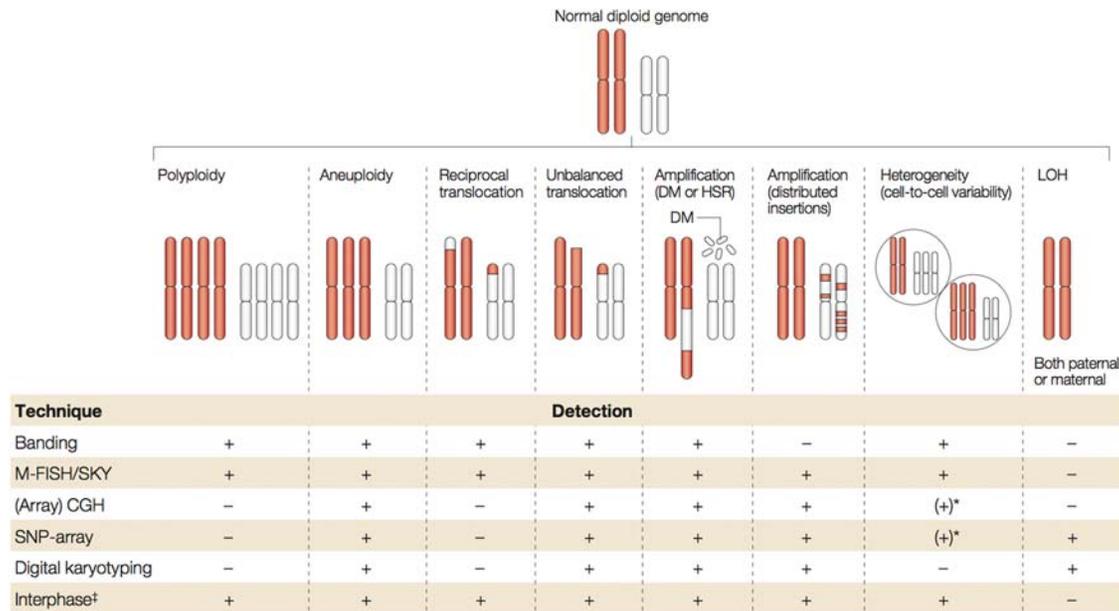
Every FISH technique has unique advantages and limitations for detecting particular genetic aberrations within clinical samples. Fig. 8 shows the complementary benefits of utilizing SKY and CGH on a hypothetical tumour karyotype, whereas Fig. 9 displays a comparison between cytogenetic techniques for identifying chromosomal abnormalities.

## Negative aspects of FISH

Fig. 9 showed that interphase FISH (depending on choice of probe) could be applied to identify all of the suggested genetic aberrations with the exception of loss of heterozygosity, demonstrating the power of the technique through its broad detection range. However, FISH techniques have a single major downfall: Interphase (and metaphase) FISH and to a lesser extent SKY and M-FISH can only detect known genetic aberrations, providing the specific probe is available (in other words a probe for a known genetic aberration has to be hybridized to the specimen in order for the FISH technique to indicate the presence or absence of that specific genetic aberration alone). FISH analysis with locus-specific probes or chromosome-specific DNA libraries is restricted to the targeted chromosome or chromosomal sub-region. Therefore, and in strong contrast to chromosome banding-based karyotype analysis, while most valuable in the confirmation of previously characterized chromosomal aberrations, FISH cannot serve as a screening test for chromosomal rearrangements since most FISH techniques can only detect known imbalances.<sup>35, 38</sup> SKY (and M-FISH) can detect multiple karyotype abnormalities simultaneously, but both techniques are dependent on combined fluorochrome probes. M-FISH and SKY are not useful for distinguishing intrachromosomal rearrangements such as duplications, deletions or inversions.<sup>32</sup>

The development of CGH partially compensates for the primary disadvantage of other FISH techniques because the main advantages of CGH is its use as a discovery tool, since it requires no previous knowledge of the chromosome imbalance that is involved.<sup>35</sup> However, CGH requires the use of the relevant tumour DNA for its application and uses metaphase chromosome spreads as targets for hybridization which can only detect relatively large gains or losses in genomic material.<sup>42</sup> Array CGH allows greater resolution, analysing DNA sequences from evenly spaced loci along the entire genome detecting smaller amplifications and deletions. In 2004, Ishkanian *et al.* produced the first sub-megabase resolution tiling set array covering the complete human genome which identified previously unknown DNA alterations including microamplifications and deletions containing oncogenes, tumor-suppressor genes and new genes that may be associated with multiple tumour types.<sup>42, 43</sup>

The limitations of the current resolution of interphase FISH was recently highlighted by Savola *et al.*<sup>44</sup> The deletion of the *CDKN2A* locus at 9p21.3, a poor prognostic indicator



**Figure 9.** Comparison of cytogenetic techniques for identifying chromosomal abnormalities. A '+' indicates that an approach is suited for identifying the chromosomal rearrangement, a '-' indicates that the aberration would be missed. In the case of heterogeneity, both array CGH and conventional CGH could detect the abnormality. Amplifications in which the amplified region is easily visible in banding analysis are noted as double minute (DM) chromosomes or homogeneously stained regions (HSR), and amplifications where the amplified region is scattered throughout the genome are noted as small insertions (distributed insertions), making identification using banding analysis impossible. Asterisk indicates that several experiments are needed. Dagger; indicates the detection rate of interphase cytogenetics depends on probe selection. Adapted from Speicher and Carter.<sup>35</sup> Reprinted by permission from Macmillan Publishers Ltd: *Nat Rev Genet* 6: 782–792, copyright 2005.

in the Ewing sarcoma family of tumours, is primarily detected by FISH using a commercial probe with an approximate size of 190 kb. Owing to limitations in resolution, FISH analysis may fail to detect microdeletions smaller than 190 kb.<sup>44</sup> In their study, array CGH was performed which revealed 9p21.3 deletions encompassing the CDKN2A locus in eight-cell lines and in six tumours. In four cases (two cell lines and two tissue samples), the deletion was less than 190 kb in size, including a 58-kb microdeletion, implicating CDKN2A FISH analysis can give false-negative results in cases with small microdeletions.<sup>44</sup>

### Conclusion

Despite the minor disadvantages discussed in the previous section, the advent of FISH in cytogenetics in has proved invaluable in both diagnostics and research. The power of its ability to identify specific genetic aberrations has propelled FISH-based techniques to the forefront of screening procedures for prenatal,<sup>45, 46</sup> paediatric<sup>47</sup> and adult cases<sup>48</sup> in a wide variety of cell types, including paraffin-embedded tissue, making FISH analysis data a useful tool in the decision of therapy to combat cancer.<sup>49</sup> This is supported by a recently conducted survey by Wordsworth *et al.*<sup>50</sup> who reported that the most common techniques used for the testing of somatic mutations in laboratories were IHC

and FISH. Most of the laboratories surveyed predicted testing would increase over the next 10 years, particularly for DNA testing using microarrays.<sup>50</sup> I agree with the results of this survey and believe the near future of FISH lies in array-based technology, either with disease-specific CGH arrays that test for every known abnormality for a particular cancer; or disease-specific microarrays that display the mRNA expression levels of any oncogene and tumour suppressor gene; or a possible hybrid of the two displaying both quantitative and qualitative data. Steps to achieving these short-term goals have already been achieved: disease-specific arrays have been constructed for cancer diagnostics for some tumour types such as chronic lymphocytic leukaemia and certain types of lymphoma<sup>51, 52</sup> and automated FISH imaging systems such as the Ariol SL-50 are already well-established in detecting patterns of genetic alterations during cancer development.<sup>53</sup>

The ultimate goal of FISH utilization would be an array-based screen using the complete oncogenic repertoire to diagnose any prenatal or postnatal aberration(s). Any mutations could be rectified accordingly using gene therapy as a method of cancer prevention. However, the prevalence of mutations in human cancers are highly variable, each with a unique assortment of abnormalities that contribute towards tumour-genesis at different developmental stages and extents. Improved aetiology through techniques such as FISH may

be crucial in the fight against cancer, with the knowledge acquired effectively directed towards the research and development of better treatment strategies to benefit the sufferers of diseases based on these genetic aberrations.

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## Author biography

Ryan graduated from Royal Holloway University of London in July 2008 where he studied a BSc. in Biochemistry. This article was based upon his combined interests for cell biology, cancer aetiology and microscopy. Since September 2008 Ryan has been working as a Scientific Officer in the Drug Development department of Paediatric Oncology at The Institute of Cancer Research. Ryan aspires to complete a PhD to help him pursue a long career in Cancer Research.

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