Molecular Cytogenetics: An Indispensable Tool for Cancer Diagnosis

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Cytogenetic aberrations may escape detection or recognition in traditional karyotyping. The past decade has seen an explosion of methodological advances in molecular cytogenetics technology. These cytogenetics techniques add color to the black and white world of conventional banding. Fluorescence *in-situ* hybridization (FISH) study has emerged as an indispensable tool for both basic and clinical research, as well as diagnostics, in leukemia and cancers. FISH can be used to identify chromosomal abnormalities through fluorescent labeled DNA probes that target specific DNA sequences. Subsequently, FISH-based tests such as multicolor karyotyping, comparative genomic hybridization (CGH) and array CGH have been used in emerging clinical applications as they enable resolution of complex karyotypic aberrations and whole global scanning of genomic imbalances. More recently, cross-species array CGH analysis has also been employed in cancer gene identification. The clinical impact of FISH is pivotal, especially in the diagnosis, prognosis and treatment decisions for hematological diseases, all of which facilitate the practice of personalized medicine. This review summarizes the methodology and current utilization of these FISH techniques in unraveling chromosomal changes and highlights how the field is moving away from conventional methods towards molecular cytogenetics approaches. In addition, the potential of the more recently developed FISH tests in contributing information to genetic abnormalities is illustrated. *(Chang Gung Med J 2012;35:96-110)*

Key words: molecular cytogenetics, fluorescence *in-situ* hybridization, multicolor karyotyping, comparative genomic hybridization, array CGH

The rationale of classifying hematological malignancies is based on the separation of diseases with distinct clincopathologic and biologic features. Recognizing the association between specific cytogenetic abnormalities and certain morphologic and clinical features, the World Health Organization has categorized four unique subtypes of acute myelocytic leukemia according to cytogenetics.[1] Therefore, cytogenetics study is currently considered a mandatory investigation in newly diagnosed leukemia

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owing to its usefulness in disease diagnosis, classification and prognostication. The vast majority of recurrent chromosomal rearrangements associated with leukemia were originally identified by cytogenetic analysis, which remains the gold standard laboratory test since it provides a global analysis for abnormality on the entire genome. Although banding techniques represent the central theme at every cytogenetics laboratory, it is sometimes difficult to karyotype the tumor cells from a patient owing to unfavorable factors such as low specimen cell yield, low mitotic index, poor quality metaphases and other technical difficulties. In addition, these techniques demand expertise such that the interpretation of variant translocations or complex karyotypic configurations may challenge even the most experienced cytogeneticist. The fluorescence *in-situ* hybridization (FISH) technique can be used to map loci on specific chromosomes, detect both structural chromosomal rearrangements and numerical chromosomal abnormalities, and reveal cryptic abnormalities such as small deletions. It has managed to overcome many of the drawbacks of traditional cytogenetics. FISH is routinely applied in the clinical laboratory and allows nearly unlimited and targeted visualization of genomic DNA using either metaphase spread, interphase nuclei, tissue sections, or living cells. FISH applications are particularly important for the detection of structural rearrangements such as translocations, inversions, insertions, and microdeletions, as well as for identification of marker chromosomes and characterization of chromosome breakpoints. FISH is essentially a molecular technique which has greatly enhanced the accuracy and efficiency of cytogenetic analysis by bringing together cytogenetics and molecular biology. The impetus for many of these FISH technology innovations has been the direct result of an increased understanding of the sequence, structure and function of the human genome, which has highlighted the intricate marvel of the DNA architectural blueprint housed within our chromosomes.[2,3] This review will summarize the development, current utilization and technical pitfalls of molecular cytogenetics techniques in clinical and research laboratories. Furthermore, this article highlights how, with advancements in technology, the study of chromosomal abnormalities is moving away from conventional methodologies towards molecular cytogenetics approaches.

Use of FISH probes in the clinical laboratory

There are a large number of good quality, directly labeled commercial FISH probes available, rendering the technology accessible to clinical laboratories. They also provide strong signal intensity with low background. The advantage of direct labeling for *in-situ* hybridization is that more than one probe may be used simultaneously with each labeled with a different fluorochrome. In the clinical laboratory, the most useful FISH probe systems are 1) centromeric probes, 2) chromosome painting probes, and 3) locus specific probes for gene fusion, gene deletion or duplication.

Centromeric enumeration probes (CEP) hybridize to the alpha (or beta) satellite repeat sequences within the centromeric regions specific for each chromosome and are used for chromosomal enumeration. CEPs are applicable in demonstration of trisomy, monosomy and ploidy level abnormalities. Chromosome painting probes are generated from chromosome-specific probe libraries. They are designed to mark the entire chromosome of interest (Fig. 1A), and are useful in deciphering cytogenetic aberrations that are difficult to resolve on morphological grounds, such as marker chromosomes of uncertain nature or complex changes.[4] However, small or cryptic rearrangements of < 2-3 megabases (Mb) will not be uncovered using these probes. Locus specific probes hybridize to a unique sequence site in the human genome. They are most frequently used to target genes of interest in order to detect rearrangements, gains, and deletions as well as amplification in both metaphase and interphase cells (Fig. 1A). Interphase analysis with FISH probes is quite an attractive and practical way to assess amplification of v-erb-b2, erythroblastic leukemia viral oncogene homologue 2 *(HER2)* in human breast cancer tissue sections, which identifies patients who might benefit from trastuzumab (Herceptin) treatment (Fig. 2A).[5] In practical terms, FISH is considered the best approach for detection of v-myc, myelocytomatosis viral related oncogene, neuroblastoma derived *(MYCN)* amplification in childhood neuroblastoma (Fig. 2B). It can distinguish between *bona fide* low levels of the *MYCN* amplification from chromosome polysomy, and copy number heterogeneity among tumor cells can be identified.[6] Interestingly, genetic heterogeneity in neuroblastoma can occur between primary tumor and bone marrow

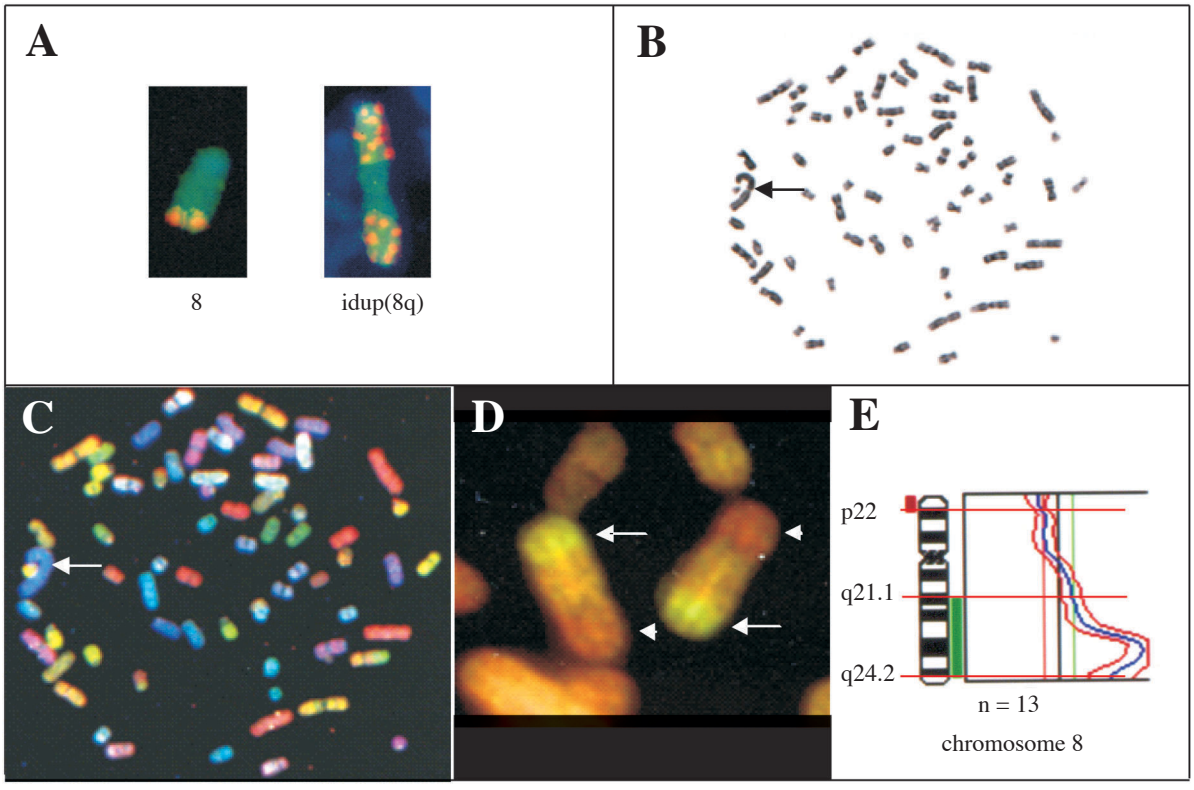

Fig. 1 Cytogenetic characterization of a cancer cell line with various FISH-based approaches. (A) Metaphase FISH using a whole chromosome painting probe (green) and *C-MYC* probe (red), which shows tandem duplication of the *C-MYC* gene on both arms of the idup(8q) chromosome (right panel) and one *C-MYC* gene on the normal chromosome 8 (left panel). (B) G-banded metaphase showing a marker chromosome (arrow). (C) SKY image showing the derivative chromosome 8 (arrow). (D) CGH analysis showing amplified 8q sequences with a green signal (arrow) and deletion of 8p sequences with a red signal (arrowhead). (E) Average ratio CGH profile of chromosome 8, showing deletion of 8p22-pter and amplified 8q21.1-q24.2.

metastasis, and has been documented by FISH analysis.[6]

There are two main systems of locus specific FISH probes for the detection of gene rearrangements.

Dual color translocation probes

The initial design of dual color translocation probes in detecting chromosomal translocations employs the dual color single fusion system (S-FISH).[7] Typically, a probe labeled with one fluorochrome spans the 5'end to the translocation breakpoint of a gene and another probe labeled with a different fluorochrome spans the 3' end of the breakpoint of the partner gene (Fig. 2C). Thus, in a metaphase or an interphase harboring the translocation, there is one signal each of the wild type allele and a fusion signal caused by juxtaposition of the fluorochromes as a result of gene fusion (Fig. 2D). However, the major drawback of the S-FISH system is the relatively high false positive detection rate owing to close migration of two chromosomes or overlap of signals by chance. This caveat is especially important in the detection of low-level clones for minimal residual disease and in monitoring for early disease relapse. In order to tackle this drawback, the dual color signal fusion with extra signal (ES-FISH) system was subsequently developed.[7] The design is essentially the same as S-FISH but with a larger probe spanning upstream and downstream of the translocation breakpoint of one of the two genes involved in the fusion, so that an extra signal (diminished fluorescent intensity) is produced if the gene is disrupted, in addition to signals of the wild type alle-

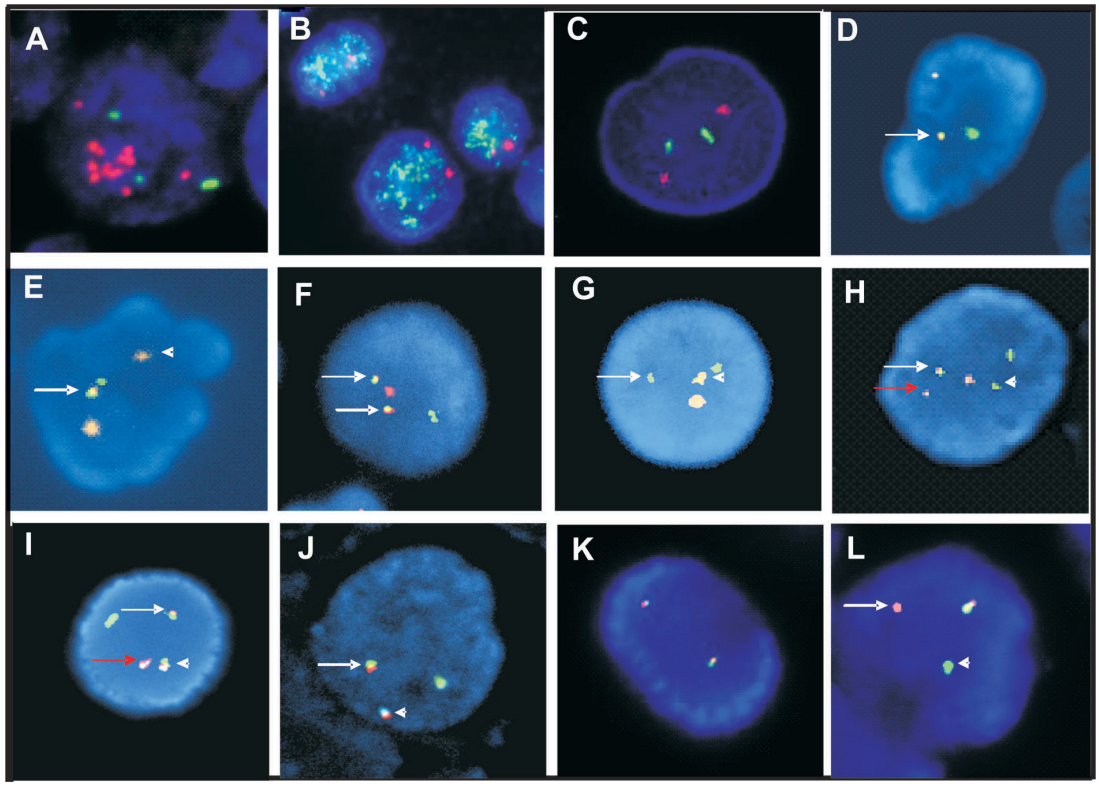

Fig. 2 FISH signal patterns of clinically used FISH probes. (A) A *HER-2* DNA probe (red) and centromeric probe for chromosome 2 (green) hybridized to breast tissue showing multiple copies of the *HER-2* gene as represented by three green and multiple red signals. The ratio of red to green probe signals is greater than 2.0 indicating *HER-2* amplification. (B) *MYCN* amplification in neuroblastoma cells. The *MYCN* gene is labeled with a green fluorochrome, while the centromeric probe for chromosome 2 is labeled with a red fluorochrome. (C) Interphase FISH with a *BCR/ABL* S-FISH probe, showing 2 green and 2 red signals in a normal cell. The *BCR* probe is labeled with a green fluorochrome, while the *ABL* probe is labeled with a red fluorochrome. (D) Interphase FISH with a *BCR/ABL* S-FISH probe, showing a fusion signal (arrow) in a Ph+ cell. (E) Interphase FISH with a *BCR/ABL* ES-FISH probe, showing a fusion signal (arrow) and extra red signal (arrowhead) in a Ph+ cell. (F) Interphase FISH with a *BCR/ABL* D-FISH probe, showing two fusion signals in a Ph+ cell (arrow). (G) Interphase FISH with a *BCR/ABL* D-FISH probe. A Ph+ cell harboring an insertion of 5'*BCR* at the *ABL* gene at 9q34 shows a fusion signal (arrowhead) and a green 3'*BCR* residual signal (arrow). (H) Interphase FISH with *BCR/ABL* D-FISH probe. A Ph+ cell harboring three-way translocation shows a fusion signal (white arrow) and two split 5'*ABL* (red arrow) and 3'*BCR* (arrowhead). (I) Interphase FISH with BCR/ABL/ASS tri-color D-FISH, showing a red/green fusion signal in the Ph chromosome (white arrow), an aqua/red signal in the normal chromosome 9 (red arrow) and an aqua/red/green fusion signal in the der(9) chromosome (arrowhead). (J) Interphase FISH with *BCR/ABL/ASS* tri-color D-FISH. A Ph+ cell harboring a *ABL/BCR* deletion in the der(9) chromosome shows a red/green fusion signal in the Ph chromosome (arrow) and an aqua/red signal in the normal chromosome 9 (arrowhead). (K) Interphase FISH with *ALK* break-apart FISH probe showing 2 fusion signals in a normal cell. The 5'*ALK* probe is labeled with a green fluorochrome, while the 3'*ALK* probe is labeled with a red fluorochrome. (L) Interphase FISH using an *ALK* break-apart FISH probe. The *ALK* gene rearranged cell shows split signals, a red signal 3'*ALK* (arrow) and a green signal 5'*MLL* (arrowhead).

les and the fusion signal (Fig. 2E). The development of the dual color dual fusion (D-FISH) probe represents a significant technological advancement in both disease diagnosis and treatment monitoring,[8] and has gained popularity. In D-FISH, large DNA probes span upstream and downstream of the translocation breakpoint of both fusion partners, so that in a positive metaphase or cell, there is one signal each for the wild type alleles and two fusion signals, one for the fusion gene and the other for the reciprocal prod-

uct (Fig. 2F).[7] D-FISH probes have a very low false-positive rate as dual fusion signal patterns rarely arise by chance. Using the strict scoring criteria, and scoring at least 300 nuclei, it is now possible to further reduce the cutoff level of false positive cells to 0.25%. When extended to the analysis of 6000 nuclei, the detection limit improved to 0.079%.

Of note, the D-FISH system can easily identify chromosomal translocation variants with atypical signal patterns. An atypical FISH pattern in chronic myelogenous leukemia (CML) due to cryptic insertion of the *BCR* to *ABL* gene at 9q34 has also been reported (Fig. 2G).[9] In addition, neoplastic cells with three-way translocation, involving 3-point breaks between three chromosomes without an *ABL-BCR* fusion, can also be identified using D-FISH probes (Fig. 2H). Interestingly, three-way translocation with a 4-point breaks mechanism and reciprocal gene fusion on the third chromosome have also been reported in acute promyelocytic leukemia (APL) and CML.[10,11] Using these atypical FISH patterns as an example, we have illustrated that in clinical practice, atypical interphase FISH should not be interpreted in isolation, and should be integrated with information gathered through conventional cytogenetics, metaphase FISH, and if necessary molecular genetic studies.[12] This point was recently emphasized when a case of cytogenetically cryptic APL was detected to harbor a *PML-RARα* fusion transcript by whole genome sequencing, which was subsequently confirmed by metaphase FISH to result from insertion of *PML* sequences from chromosome 15 into *RARα* on chromosome 17 that led to a classic bcr3 *PML-RARα* fusion gene.[13]

Loss of DNA around the breakpoints of translocation has been observed in hematologic malignancies. Notably deletions of derivative chromosome 9 involving whole or part of the reciprocal *ABL-BCR* fusion have been documented in CML using the D-FISH system.[14] These deletions, found in around 15% of patients with CML, are large and occur at the time of the Ph translocation.[14] Once the deletion is identified, the D-FISH system is relegated to S-FISH and no longer suitable for residual disease detection since chance signal juxtaposition is not readily discriminated from fusion. To circumvent the problem, a new method that incorporates an aqua-labeled probe for the *ASS* gene into the *BCR-ABL* D-FISH probe set was subsequently introduced (Fig. 2I).[15] This tricolor D-FISH method takes advantage of the *ASS* probe to distinguish between overlapping *BCR* and *ABL* signals due to chance juxtaposition in normal cells and genuine *BCR-ABL* fusion signals in neoplastic cells (Fig. 2J).[15]

Dual color break-apart probes

For genes such as *MLL, TEL* and *RARα* that show multiple translocation partners, the use of break-apart FISH probes conveniently gives important information on gene rearrangement, albeit unable to specifically incriminate the partner gene.[16] Typically, a probe labeled with one fluorochrome spans the 5'end of the translocation breakpoint while another probe labeled with a different fluorochrome spans the 3' end. The expected number of spots in a normal interphase nucleus is two fusion signals (Fig. 2K). In rearrangements involving the gene region, the observed pattern will be one fusion signal and two split signals (Fig. 2L). The probe will identify gene deletions as signal fusion and the loss of the other fusion signal, consistent with preservation of one allele and deletion of the other.[17] Furthermore, it will also identify gene amplification or duplication of the corresponding chromosome band that includes the wild-type gene.[18] The copy number of the fusion signal will be increased (> 2) in these cases.

Taken together, FISH has proven to be an essential tool that can be incorporated in most cancer cytogenetics laboratories. It is sensitive, rapid and serves as an indispensible complement to conventional cytogenetics. Issues related to analytical sensitivity should be considered, especially with respect to disease monitoring for post-treatment samples. It is advisable to subscribe to external quality assurance or proficiency testing programs, such as that operated by the College of American Pathologists, that can cater to laboratories performing FISH studies. A molecular cytogenetics laboratory must establish standards for analysis and interpretation that comply with accreditation standards and that are appropriate for that laboratory.

Superiority of FISH assay

FISH versus cytology

Bladder cancer is most frequently diagnosed when investigating hematuria. Urine cytology is performed on a urine sample which is centrifuged and the sediment is examined under the microscope to

detect abnormal cells that may be shed into the urine by a cancer. However, many early bladder cancers may be missed by this test and hence a negative or inconclusive test does not effectively rule out bladder cancer. Increased chromosomal instability and aneuploidy are characteristic of bladder tumor progression. Using the FISH method, a mixture of CEP 3, CEP 7, CEP 17, and locus specific identifier p16 FISH probes is used to enumerate chromosomes 3, 7, and 17 and detect the 9p21 locus deletion on chromosome 9, which is a non-invasive strategy for bladder cancer screening.[19]

FISH versus immunohistochemistry

Since patients with *HER2* amplified breast carcinoma who receive trastuzumab (Herceptin) have improved clinical outcomes, accurate *HER2* testing is essential for quality patient care. Clinical practice guidelines for *HER2* testing in breast cancer have recently been published.[20] In practice, the *HER2* status can be determined by immunohistochemistry (IHC) and FISH methods. IHC detects HER2 transmembrane protein by using a labeled antibody. Studies show that non-amplified cells with negative IHC staining have less than half of the receptors compared with those with *HER2* gene amplification.[21] A category of intermediate HER2 expression status (2+) exists with staining intensity -between that of clear-cut negative (0 and 1+) and positive (3+), and these cases should undergo FISH to document the *HER2* status. Currently, based on the more tedious nature and higher cost of FISH, most countries have recommended two-step testing for *HER2* status, with FISH used to confirm 2+ IHC staining.[22]

FISH versus conventional cytogenetics

Although translocation (15;17) (q22;q21) and *PML-RARα* fusion are regarded as highly specific for APL, t(15;17) (q22;q21) not associated with APL and negative for *PML-RARα* rearrangement has been reported.[23,24] Detailed molecular analysis showed no evidence of *PML-RARα* rearrangement, thus confirming that the translocation breakpoints in this patient did not involve the *PML* and *RARα* genes. These observations showed that morphologic, cytogenetic and FISH/molecular features must all be considered for an accurate diagnosis of APL. These cases highlighted the importance of combined modalities.

FISH versus molecular biology

The advantage of FISH is for the detection of chromosomal translocations that are not amenable to polymerase chain reaction (PCR) detection due to scattering of breakpoints throughout the gene, such as *CBFβ* rearrangement. FISH probes are much larger and hence there is better coverage of non-clustered potential breakpoints than in PCR analysis.

Variant transcripts generated from classic translocations may also cause diagnostic dilemmas. We encountered a rare case of p230 CML in which the e19a2 *BCR-ABL* fusion transcript level was underestimated by a commercial real-time quantitative PCR (RQ-PCR) reagent kit (Fig. 3). In our patient the RQ-PCR result was disproportionately lower than that obtained by FISH (Table). Since a commercial RQ-PCR system targets the M-bcr breakpoint catering for the classic b2a2 and b3a2 transcripts, the presence of a variant e19a2 transcript may produce an underestimated or even falsely negative result, as the primers are spaced widely apart, thus lowering the PCR efficiency.

Multicolor FISH

Multicolor FISH is based on the simultaneous hybridization of 24 chromosome-specific composite probes. Multicolor FISH is suitable for identification of cryptic chromosomal aberrations, such as the translocation of telomeric ends, which is difficult to detect using conventional cytogenetics alone, and the identification of unidentified (marker) chromosomes, and unbalanced chromosomal translocations that remain elusive after conventional cytogenetics analysis. These chromosome-painting probes are generated from flow-sorted human chromosomes. Chromosome-specific unique colors are produced by labeling each chromosome library either with a single fluorochrome or with specific combinations of multiple fluorochromes (combinatorial labeling). Two multicolor fluorescence technologies have been introduced, multiplex FISH (M-FISH) and spectral karyotyping (SKY).[25,26] The difference between the two techniques is in the image acquisition process; they employ different methods for detecting and discriminating the different combinations of fluorochromes after *in-situ* hybridization. In SKY, image acquisition is based on a spectral imaging system using an interferometer and a charge-coupled device camera (Fig. 1B & C). This makes possible the mea-

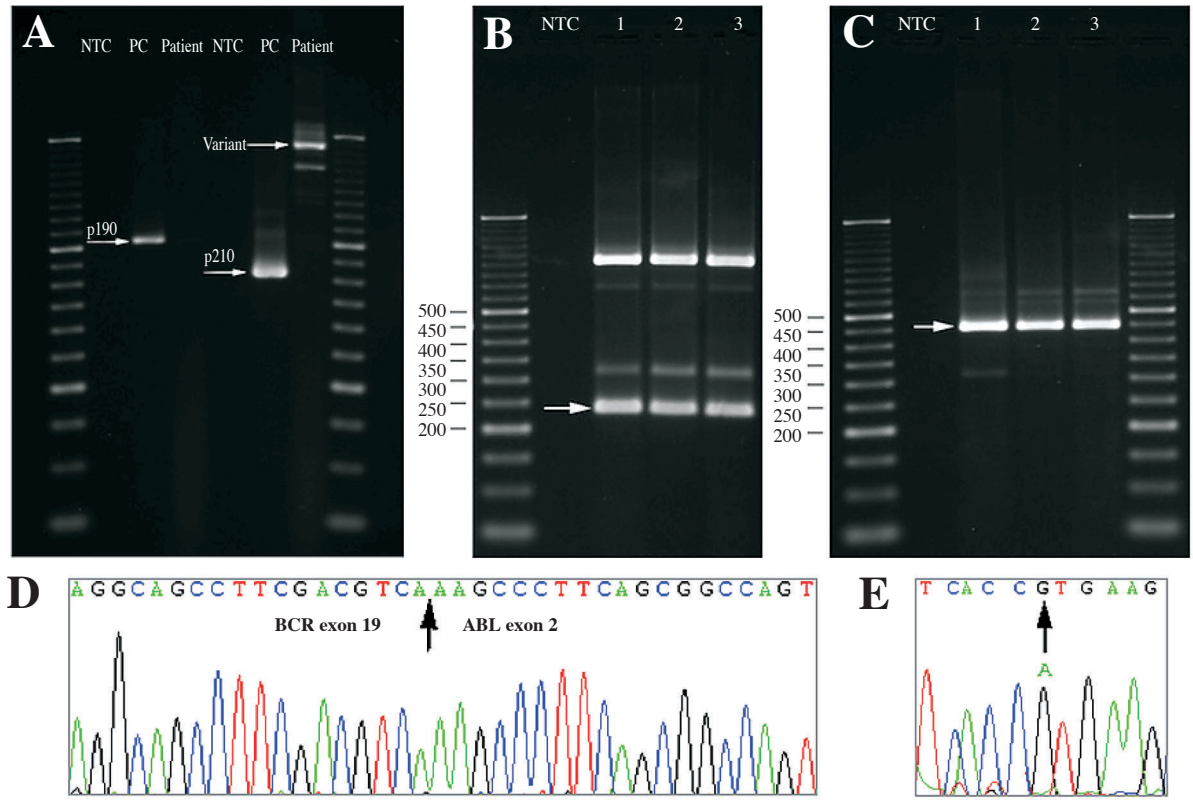

Fig. 3 Molecular analysis of p230 chronic myeloid leukemia. (A) Conventional RT-PCR detection of p190 and p210 *BCR-ABL* transcripts in accordance with the BIOMED-1 protocol, showing a large variant band. (B) Multiplex PCR for detection of atypical *BCR-ABL* fusion transcripts, showing a band of 234 bp corresponding to e19a2. (C) RT-PCR targeting the e19a2 *BCR-ABL* fusion transcript. (D) Automated sequencing confirming the e19a2 *BCR-ABL* fusion transcript. (E) Automated sequencing showing the M244V (nt.730 A→G) KD mutation. Abbreviations used: NTC: negative test control; PC: positive control; Sample 1: before imatinib; Sample 2: six months after imatinib; Sample 3: twelve months after imatinib.

Table Molecular Monitoring of A Patient with e19a2 Chronic Myeloid Leukemia

Time with respect to imatinib treatment	Hb (g/dL)	WBC (x 10^9/L)	Plt (x 10^9/L)	Cytogenetics	FISH	*BCR-ABL/ABL* ratio (IS)
Before commencement	11.1	66.1 (2% blasts)	920	Ph, inv(9)	100%	0.061
6 months	12.9	4.4	279	Not done	28.7%*	0.012
12 months	12.5	5.7	205	Not done	3.7%*	0.0075
18 months	12.6	7.1	284	Not done	Not done	0.0081
25 months	10.2	22.0 (62% blasts)	101	Frank haematological relapse in blastic phase and detection of M244V KD mutation		

Abbreviations: Hb: hemoglobin; WBC: white blood cell count; Plt: platelet count; FISH: fluorescence in-situ hybridization; IS: international scale; KD: kinase domain; Ph: philadelphia chromosome; inv(9): inv(9)(p11q13); *: FISH monitoring was performed on peripheral blood cells, whereas at diagnosis it was performed on archival bone marrow cells preserved in Carnoy's fixative.

surement of the entire emission spectrum with a single exposure at all image points and simultaneously measures the intensity for each pixel in the image at many different wavelengths.[27] In M-FISH, separate images are captured for each of the six fluorochromes using narrow bandpass microscope filters. The images are subsequently merged by dedicated software.[28]

Although the accuracy of SKY is shown to be high with an average misclassification error of 1.3%, an error of even a few pixels could lead to an incorrect cytogenetics conclusion.[29] Our previous study showed that single fluorochrome labeled whole painting probes were more sensitive than SKY probes in a case of APL with cryptic *PML-RARα* gene fusion.[29] There are two possible explanations. Firstly, different labeling methods may account for the difference in sensitivity. A whole painting probe is labeled with a single fluorochrome, whereas SKY probes are labeled with mixtures of five fluorochromes. Therefore, the resolution of a whole painting probe may be better than SKY painting probes. Secondly, in terms of detection, green and red fluorescence signals are optimal. Therefore, a whole painting probe may detect the interstitial insertion of a small chromosomal fragment or single gene into another chromosome with greater ease than SKY.[27] Nevertheless, the limitations of these techniques include the inability to detect intrachromosomal aberrations such as inversions, duplications and deletions.[28,29] Furthermore, color blending can cause the formation of additional visible bands at sites where chromosomes overlap and at translocation breakpoints. FISH analysis is subsequently required to characterize whether these bands represent small insertions or are just the result of color blending.

More specific multicolor FISH tests have been developed to facilitate the identification of an intrachromosomal rearrangement, such as cross-species color segmentation FISH (Rx-FISH) or use of human overlapping microdissection libraries that are differentially labeled (multi-color banding, mBAND).[30,31] These two techniques provide precise information on intra-chromosomal rearrangements and exact breakpoint mapping. Rx-FISH consists of the combinatorial labeling of probe sets made from the chromosomes of two gibbon species (*Hylobates concolor* and *Hylobates syndactylus*) and their hybridization to human metaphases. The success of this cross species color banding depends on a close homology (> 98%) between host and human conserved DNA, divergence of repetitive DNA, and a high degree of chromosomal rearrangement in the host relative to the human karyotype. Hybridization of human chromosomes with painting probes derived from both gibbon species showed that with the exception of human chromosomes 15, 18, 21, 22 and the sex chromosomes, each chromosome was differentiated in at least two and up to six segments. Rx-FISH relies on color combinations arising from three fluorochromes, which provides 7 colors instead of the 24 colors of M-FISH or SKY. Although this number of colors means that many chromosomal regions share the same color, the distribution of colors gives unique "color bar code" banding patterns for each homologous chromosome pair. These unique banding patterns help overcome color limitations and at the same time provide a guide to the localization of chromosomal breakpoints. To improve the resolution of the color banding technique, human overlapping microdissection libraries that are differentially labeled can be used as probes (Fig. 4). Currently, mBAND reveals a banding pattern with approximate 550 bands in the normal haploid human karyotype. The striking advantage of mBAND over Rx-FISH approaches which use individually labeled yeast artificial chromosomes or bacterial artificial chromosomes (BAC) is obvious: in order to obtain the same number of color bands, with the unique mBAND technique the complexity of the probe cocktail as well as the number of fluorochrome combinations is less than one-third that of the Rx-FISH approach.

Distinct 3-dimensional organizations of chromatin in different tissue types should be addressed, as high-order chromatin arrangements are likely to have fundamental implications for development and cell differentiation. Therefore, more advanced and sophisticated software and hardware platforms are needed to carry out multicolor 3D-FISH analyses in a high-throughput format.[3]

Comparative genomic hybridization (CGH)

CGH is a molecular cytogenetics technique based on quantitative two color FISH (Fig. 1D & E).[32] In a single experiment, CGH detects genomic imbalances in solid tumors or any desired test genome, and determines the chromosomal map position of gains and losses of chromosomes or chromo-

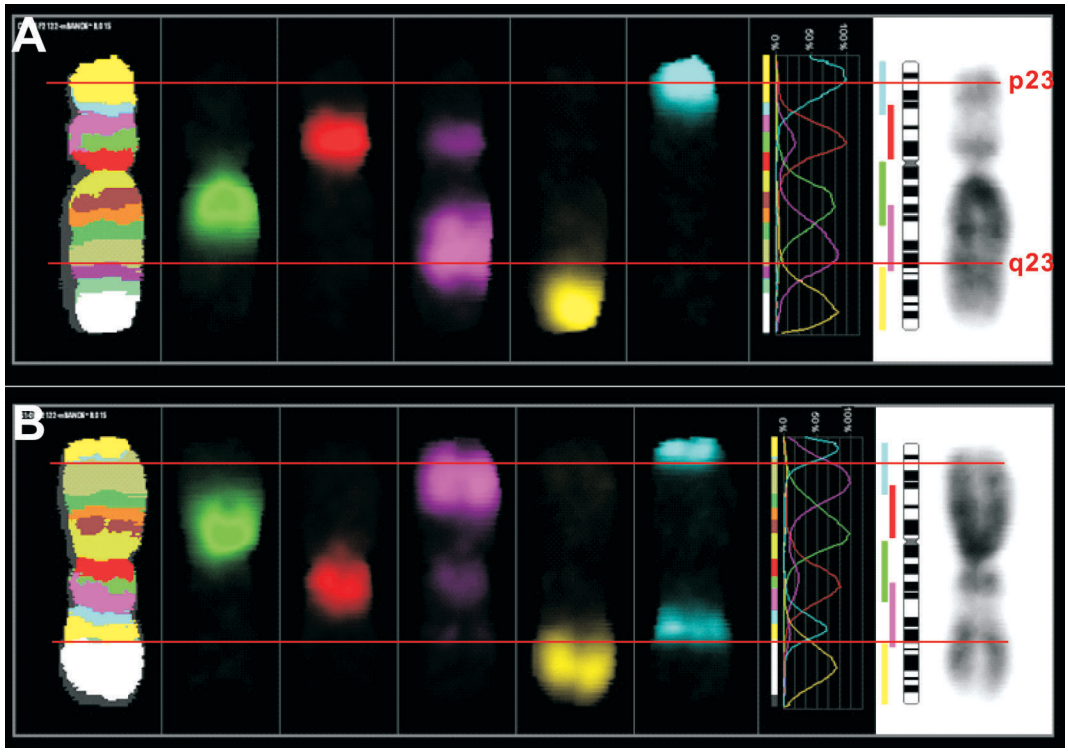

Fig. 4 Characterization of pericentric inversion chromosome 6 using a multicolor banding (mBAND) approach. (A) An mBAND pattern of normal chromosome 6. (B) An mBAND pattern of inv(6)(p23q23).

somal sub-regions on normal reference metaphase preparations using a small amount of DNA. Briefly, tumor DNA (labeled green) and normal reference DNA (labeled red) are competitively hybridized to normal human metaphase spread. The reference DNA serves as a control for local variations in the ability to hybridize to target chromosomes. The relative amounts of tumor and reference DNA bound at a given chromosome are dependent on the relative abundance of those sequences in the two DNA samples. Digital image analysis gives a measurement of the ratio of green-to-red fluorescence along the chromosome on the reference metaphase spread, reflecting the copy number of the corresponding sequences in the tumor DNA (Fig. 1D). If chromosomes or chromosomal subregions are present in identical copy numbers in both the reference and the tumor genome, the observed fluorescence is a blend of an equal contribution of red and green fluorescence. If chromosomes are lost or chromosomal sub-regions deleted in the tumor genome, the resulting color is shifted to red. A gain of a certain chromosome in the tumor would be reflected by more intense green staining on the respective chromosome in the reference metaphase preparation (Fig. 1E). Subsequently, a rapid approach, termed comparative expressed sequence hybridization, which gives a genome-wide view of relative expression patterns within tissues according to chromosomal location, has been described.[33]

CGH has become one of the most widely used cytogenetics techniques in both basic research and molecular diagnostics. A distinct advantage of CGH is that only tumor DNA is required for this molecular cytogenetics analysis. Thus, archived, formalin fixed and paraffin embedded tissue can be used as well. It is applicable to cancer research, especially for the low mitotic index of malignant cells and poor chromosome morphology and resolution. The scope of CGH has been extended to include the analysis of small amounts of DNA that have been obtained from target lesions in a specimen, such as microdissected tumor samples. This allows one to establish a correlation of the microscopic phenotype and the geno-

type in solid tumors. In addition, CGH offers a new experimental approach to study chromosomal aberrations that occur during solid tumor progression. The validity of CGH to delineate complex genetic changes in solid tumors has been investigated in several studies. However, the use of CGH is limited in for the detection of chromosomal aberrations that do not involve genomic imbalances, such as inversions and balanced chromosome translocation. Currently, the CGH technique achieves a resolution at the level of 2-4 Mb, provided that the experimental protocols are optimal. Another constraint of CGH is that ploidy aberrations escape detection by this technique.

Array CGH

The development of array CGH technology for 'molecular karyotyping' with a resolution of 100 kilobases (kb) to 1 Mb is an example of the tremendous technical advances in cytogenetics that have changed clinical diagnostic and research approaches.[34] The concept and methodology of array CGH (also called matrix CGH) is essentially the same as its traditional predecessor except that the template against which the genomic comparison is performed is no longer a normal metaphase spread. Array-based CGH greatly improves the resolution of the technique by substituting the hybridization targets, the metaphase chromosome spread, with genomic segments spotted in an array format. In order to comprehensively assess the genome and to identify the focal genetic events occurring during tumorigenesis, a whole genome tiling path array CGH approach must be employed. The genomic segments can be BAC or P1 artificial chromosome clones for hybridization targets immobilized on glass slides as arrays. DNA arrays consisting 2,000 to 4,000 BAC clones representing the sequenced genome at approximately 1 Mb intervals have been developed.[35] Using overlapping clones, the resolution of the array was increased beyond the size of a single BAC clone and gains and losses of regions as small as 40-80 kb are detectable (Fig. 5). Oligonucleotide arrays are also used in copy-number detection. These arrays contain

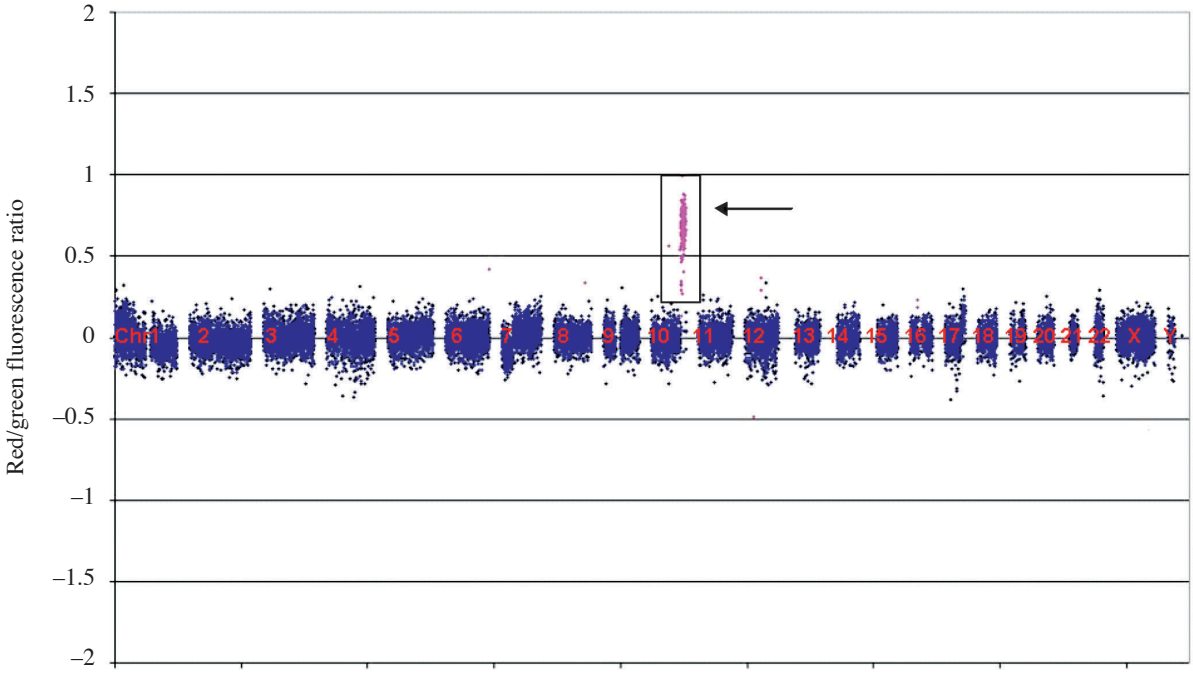

Fig. 5 A 1 Mb whole genome tiling path array CGH approach shows amplification of 150 BAC clones in the long arm of chromosome 10 from 116383658.5 to 134016426.5 in the genome (arrow). Amplification or deletion of other chromosomal sub-regions is not detected. Test DNA was labeled in red and normal reference DNA was labeled in green.

25-mer oligonucleotides originally designed to assess human single-nucleotide polymorphisms. This method has the advantage of measuring allelic loss of heterozygosity. Alongside copy-number changes using the same platform, and it allows a sensitive and specific detection of single copy number changes at the submicroscopic level throughout the entire human genome.[35] More recently, copy number changes at a resolution of individual exons have been identified.[36]

Array CGH technology has much greater multiplexing capabilities than targeted FISH studies and offers much higher resolution for overall genomic screening than conventional cytogenetics studies; array CGH allows the recognition of deletions and duplications in the genome in a single experiment. Array CGH has been used for a variety of approaches. Many of the applications are pertinent to cytogenetics laboratories, such as determination of general polymorphisms, characterization of acquired genetic changes, prenatal diagnostics, identification of congenital genetic defects, and evolutionary characterization. Recently, ultra-high resolution array painting facilitates breakpoint sequencing of the derivative chromosome has been demonstrated, and therefore the precise breakpoint region can be easily mapped.[37] Furthermore, array CGH has provided important insights into aspects of normal genomic variation. Array CGH is one of these technologies that have recently revealed a newly appreciated type of genetic variation: copy number variation (CNV), in which thousands of regions of the human genome are now known to vary in number between individuals.[38] Some of these CNV regions have already been shown to predispose to certain common diseases, and others may ultimately have a significant impact on how each of us reacts to certain foods, microscopic infections, medications, and other aspects of our ever-changing environment. Therefore, before applying array CGH in a diagnostic setting, a better knowledge of polymorphisms present in general populations is required. More recently, cytogenetically balanced translocations have in fact been frequently associated with segmental gain or loss of DNA in prostate cancer cell lines.[39] This reveals that imperfectly balanced translocations in tumor genomes are a phenomenon that occurs at frequencies much higher than previously demonstrated.[39]

The resolution and coverage of array CGH are dependent on the density of the array used. An array covering the entire genome at very high resolution would have potential advantages in clinical and research use. However, the use of more array probes is likely to generate a higher number of false positives. Quality control, fabrication, and interrogation are expensive with large arrays. Large and very high resolution arrays are likely to generate information that may be difficult to interpret. Alterations in regions of the genome that do not have established clinical relevance will be burdensome for clinical cytogeneticists for interpret usefully. Furthermore, this technique will not detect balanced rearrangements and low-level mosaicism for unbalanced numeric or structural rearrangements, and it does not exclude mutations in any gene represented on the array clones.

The complexity of genomic aberrations in most human tumors hampers delineation of the genes that drive tumorigenesis. Recently, cognate mouse models which recapitulate these genetic alterations with unexpected fidelity have been demonstrated.[40] These results indicate that cross-species array CGH analysis is a powerful strategy to identify responsible genes and assess their oncogenic capacity in the appropriate genetic context.[40]

FISH and personalized medicine

Personalized medicine is the tailoring of medication to individual patients according to genetic variation. By virtue of its ability to detect drug targets, the FISH technique is a convenient method to support the practice of personalized medicine. In addition to guiding Herceptin treatment in breast cancer with HER2 FISH testing, as mentioned above, numerous other examples can be found in hematological malignancies and solid tumors. In chronic lymphocytic leukemia, risk stratification can be undertaken by a FISH panel and coupled with determination of IgV_H mutation status or expression of ZAP70 and CD38. The presence of del(11q) and del(17p) is often associated with a poor prognosis, del(13q) or a normal karyotype is associated with low-risk disease, and the presence of trisomy 12 may be considered a marker of intermediate risk.[41] Likewise, recent international guidelines recommend a minimum FISH panel for the detection of t(4;14), t(14;16) and del(17p) which recognizes the high risk category in myeloma.[42] The interphase FISH test

should be performed on a bone marrow sample enriched for abnormal plasma cells for analysis of genetic aberration. First reported in 2007, the *EML4-ALK* gene fusion is a new molecular aberration in non-small cell lung cancer (NSCLC) and occurs as a result of a small inversion within chromosome 2p.[43] The fusion gene is oncogenic and represents a novel molecular target in NSCLC. Patients tend to be younger, are more likely to be male, have never smoked or are light smokers and are double negative for *EGFR* and *KRAS* gene mutations.[44] *EML4-ALK* fusion may be detected by a dual-colour split-apart FISH probe that targets the *ALK* gene (Fig. 2L). Patients harboring *EML4-ALK* gene fusion are candidates for clinical trials of ALK inhibitor therapy.

Future prospects and concluding remarks

In the past decades, innovative technical advances in the field of cytogenetics have greatly enhanced the detection of chromosomal alterations and have facilitated the research and diagnostic potential of cytogenetics studies in constitutional and acquired diseases. FISH plays a central role in combination with karyotyping to rapidly detect and verify specific chromosomal aberrations. The field of molecular cytogenetics has expanded beyond the use of FISH to other techniques that are based on the principle of DNA hybridization. The considerable gap in resolution conventional cytogenetics techniques (5-10 Mb pairs) and molecular biology techniques (base pairs) has been bridged to a large extent by FISH, which allows the assessment of genetic changes on chromosome preparations. Some noteworthy innovations that have altered the landscape of clinical and research investigations include the use of various targeted FISH techniques, multicolor FISH to identify chromosomal alterations unresolved by karyotyping, and chromosomal CGH which offers genome wide-screening by determining DNA content differences and characterizing chromosomal imbalances even when fresh specimens and chromosome preparations are unavailable. More recently, the development of array CGH, which allows the detection of much smaller genomic imbalances, involves the use of an ordered set of defined nucleic acid sequences derived from various sources, immobilized on glass slides.

Currently, the array CGH approach is poised to revolutionize modern cytogenetic diagnostics and provide clinicians with a powerful tool in their diagnostic armamentarium. The cross-species array CGH studies described testify to the notion that genetically tractable mouse models represent an invaluable tool not only to identify new cancer-causing genes but also to assess the context-dependent vulnerability of tumors to multi-target intervention strategies.[40] However, all gains and losses identified on the array CGH should be validated by FISH or molecular confirmation analysis.

Taken together, the goal of the molecular cytogenetics laboratory is to identify the type of techniques that are most useful and informative for a particular study, prepare quality experimental materials, and perform a thorough analysis to arrive at an interpretation that is useful for research and diagnostic purposes.

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分子細胞遺傳學：不可或缺的癌症診斷工具

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在常規細胞遺傳學的檢查中，有很多染色體的變異都未能被檢測或辨認出來。在過去十年裡，分子細胞遺傳學技術在方法上都有突破性的發展，細胞遺傳學的檢查從而由黑白走向色彩。在白血病和腫瘤的基礎和臨床的科研，以及疾病的診斷上，螢光原位雜交技術 (FISH) 的檢測都突顯了它是一種不可或缺的工具。FISH 是利用螢光顏料標記在相關特殊核酸序列的探針，來進行檢測染色體的異常。隨後在 FISH 基礎上再開發的技術：例如，多色核型分析、比較基因組雜交 (CGH) 技術和陣列比較基因組雜交 (array CGH) 技術等，分別應用于臨床上來檢查複雜染色體的變異和篩檢全基因組的數量失衡。最近新發展的異種 array CGH 技術更提升了在尋找致癌基因的能力。FISH 的檢測結果往往影響臨床上的決策，尤其在血液病的診斷、評估預後和治療上；也同時允許了個人化治療的實踐。本文綜述了 FISH 技術在闡釋染色體改變時的應用和方法，並且分析了細胞遺傳學採用分子技術的進程。除此以外，本文也對新開發的 FISH 檢查在遺傳異常上所提供的資料作出探討。(長庚醫誌 2012;35:96-110)

關鍵詞： 分子細胞遺傳學，螢光原位雜交，多色核型分析，比較基因組雜交，陣列比較基因組雜交

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