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Development and Clinical Application of an Innovative Fluorescence in situ Hybridization Technique Which Detects Submicroscopic Rearrangements Involving Telomeres

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Abstract
We report an innovative fluorescence in situ hybridization technique which exploits a unique resource of 41 telomere-specific probes and allows the simultaneous analysis of the subtelomeric region of every chromosome for deletion, triplication and balanced translocation events. This technique requires only a single microscope slide per patient and is expected to be a useful diagnostic tool with applications in the fields of idiopathic mental retardation, the detection of congenital abnormalities and in some forms of cancer. This will lead to more accurate genetic counselling of patients and their families and will provide the basis for future diagnostic, therapeutic and preventative measures.

Introduction

Standard cytogenetic techniques enable all human chromosomes to be stained and identified individually by their unique banding patterns. Routine cytogenetic analysis has a 400–500 band resolution and is capable of detecting rearrangements involving 5–10 Mb DNA depending on the chromosomal region. Higher resolution analysis of 850–1,000 bands is possible, but this is time-consuming and only useful when searching for suspected rearrangements in known chromosomal locations rather than for screening the entire genome. Consequently, the detection of subtle rearrangements in patients who are suspected to have a chromosomal anomaly, but for whom there are no clues regarding the chromosomal origin, is problematical.

The advent of fluorescence in situ hybridization (FISH) technology has made new approaches available for cytogenetic screening [1, 2]. Whole chromosome painting and reverse chromosome painting have facilitated the identification of some subtle rearrangements, but regions such as telomeres are often underrepresented and subtle deletion events or structural changes within the same chromosome cannot be detected [3–5]. In addition, the ability of whole chromosome painting probes (WCPP) to detect small translocations remains uncertain. FISH using chromosome-specific unique sequence probes is suitable for detecting submicroscopic deletion or duplication events, but these probes are not amenable for use in a routine screening test because they exclude significant tracts of the genome where submicroscopic rearrangements may occur. However, there is evidence that certain regions of

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Table 1. Cosmid, P1 and PAC clones used in current studies

Telomere	Clone name	Clone type	Reference
1p	CEB108	Cosmid	7
1q	2123.2a1	Cosmid	This report
2p	2052f6	Cosmid	8
2q	210E14	P1	8
3p	B47a2, B47b2	Cosmid	8, this report
3q	B35c1	Cosmid	8
4p	B31	Cosmid	13
4q	cT55	Cosmid	14
5p	84C11	Cosmid	8
5q	B22a4	Cosmid	8
6p	36I2	PAC	8
6q	2158e3	Cosmid	8
7p	109A6	Cosmid	8
7q	2000a5	Cosmid	8
8p	2205a2	Cosmid	8
8q	2053b3	Cosmid	8
9p	34H2	PAC	8
9q	2241c1	Cosmid	This report
10p	2189b6	Cosmid	8
10q	2136a1	Cosmid	This report
11p	2209a2	Cosmid	8
11q	2072c1	Cosmid	8
12p	90I5	PAC	8
12q	2196b2	Cosmid	8
13q	2002e1	Cosmid	8
14q	2006a1	Cosmid	8
15q	154P1	PAC	8
16p	cGG4	Cosmid	8
16q	D3b1	Cosmid	8
17p	2111b1	Cosmid	This report
17q	B37c1	Cosmid	8
18p	52M11	P1	8
18q	2050a6	Cosmid	8
19p	F20643	Cosmid	8
19q	F21283	Cosmid	8
20p	2005a4	Cosmid	8
20q	204A16	PAC	This report
21q	C9a1	Cosmid	8
22q	N85A3	Cosmid	15
XpYp	CY29	Cosmid	16
XqYq	c8.2/1	Cosmid	17

the genome are enriched for rearrangements. We have recently shown that as many as 8% of idiopathic mental retardation (IMR) referrals possess submicroscopic rearrangements involving telomeres and the associated subtelomeric DNA [6, 7]. Thus, the development of a FISH-based technique capable of detecting all deletions, triplications and balanced translocation events involving telomeres and subtelomeric regions would be a useful diagnostic tool.

We have recently reported a complete set of telomere-specific cosmid, P1 and PAC FISH probes capable of detecting rearrangements of subtelomeric DNA [8]. Standard FISH techniques, which involve the individual testing of every telomere probe for 24 different chromosomes, would be impractical and too costly for introduction as a routine procedure. Thus, we have developed an innovative FISH technique which requires only a single microscope slide per patient and allows the simultaneous analysis of the subtelomeric region of every chromosome for deletion, triplication and balanced translocation events. We have taken a device originally developed for multiple WCPP on a single slide [12] and incorporated it in the development of a technique specifically designed for the multiple hybridization of cosmid, P1 and PAC clones. The technique requires no novel or expensive equipment or materials, thus making it a straightforward and feasible proposition for all cytogenetic diagnostic laboratories equipped for FISH studies.

Materials and Methods

To demonstrate the diagnostic potential of the innovative FISH technique, 4 test cases were selected for analysis: (1) a phenotypically and cytogenetically normal male; (2) a phenotypically and cytogenetically normal female; (3) an IMR male patient originally reported as cytogenetically normal, but known by Southern analysis to exhibit a de novo deletion of a hypervariable probe mapping to the p-arm telomere of chromosome 1 [7], and (4) an IMR female originally reported as cytogenetically normal using conventional techniques, but shown by Flint et al. [6] to have a deletion of subtelomeric 22q and a trisomy of subtelomeric 9q.

The reliability of the technique was tested in 20 separate hybridization experiments each using fixed chromosome preparations from different individuals.

Extraction of Cosmid, P1 and PAC DNAs and Nick Translation Labelling

The cosmid, P1 and PAC telomere-specific clones used in this study are given in table 1. Cosmid, P1 and PAC DNAs were prepared using a standard caesium chloride method [9] and nick translation labelling of the prepared DNA was also performed according to standard protocols [10]. All p-arm probes were labelled with biotin-16-dUTP (Boehringer Mannheim) and all q-arm probes with digoxige-

nin-11-dUTP (Boehringer Mannheim). Following the removal of unincorporated nucleotides by passage through Sephadex G-50 columns equilibrated in 3 × SSC, the probes were diluted to give a final concentration of 10 ng/μl containing 25% sonicated salmon sperm (2 mg/ml stock) in a volume of 100 μl. With the exception of the chromosome 19 telomere-specific cosmids, 62.5 ng human COT-1 DNA (1 mg/ml; Gibco-BRL) and 150 μl dH_2O were added to each of the cosmid probes and 125 ng COT-1 DNA and 87.5 μl dH_2O to each of the PAC and P1 probes. For the chromosome 19 cosmid probes, 475 ng COT-1 DNA were added. The probe preparations were then stored at –20 °C.

Preparation of 'Template microscope slides' and 'Multiprobe Devices' for FISH

The FISH technique which we have developed was conceived from an approach originally devised for use with whole chromosome paints [11, 12]. It employs (1) a 'template microscope slide' demarcated into 24 squares (3 rows of 8 squares) labelled from 1 to 22, X and Y and (2) a 'Multiprobe device' which is essentially a coverslip with 24 raised square flat-surfaced panels (fig. 1).

Fixed chromosome suspensions were prepared directly from peripheral blood and from lymphoblastoid cell lines of the male and female normal control and male IMR patient samples according to standard protocols [10]. For each test sample, 2 μl of fixed chromosome suspension were pipetted directly on to a clean Superfrost slide (BDH). When dry, the spot was examined by phase-contrast microscopy and the number of cell nuclei and metaphases noted. The concentration of the fixed chromosome suspension was then adjusted until a 2-μl drop contained at least 15 good quality metaphases in the central area and optimally, > 300 interphase nuclei. 2-μl drops of the chromosome suspension were then added to each of the demarcated squares of a clean, labelled template slide. When dry, each slide was stored at –20 °C in a sealed light-proof slide box containing silica gel (BDH) as dessicant prior to hybridization.

The 'Multiprobe devices' were prepared by adding 1.25 μl of the probe preparations (2.3 μl for the chromosome 19 probes) to the appropriate panels of the Multiprobe device and allowing them to dry (Patent pending). For example, the p and q telomere probes for chromosome 1 were applied to the raised square to be matched with Square 1 on the template microscope slide and those for chromosome 2, applied to the raised square to be matched with Square 2 on the template slide, etc. For the 5 acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) only q-arm probes were applied. Furthermore, as the pseudoautosomal regions of the sex chromosome telomeres are identified by the same pair of p- and q-arm probes, both were applied to the 'X' square of the device leaving the 'Y' square free. The prepared device was then stored at room temperature (RT) in a light-proof container until ready for use.

FISH Protocol

The FISH strategy which was employed is shown in figure 2. Immediately prior to hybridization, each prepared template slide was equilibrated at RT and rinsed in 2 × SSC for 3 min before being dehydrated through an ethanol series of 70, 95 and 100% ethanol each for 3 min. The slide was then placed chromosome spread uppermost on a 37 °C hot block. A prepared Multiprobe device was also placed probe uppermost on the 37 °C and 2 μl hybridization solution (65% formamide (Fluka), 1 × SSC, 10% dextran sulphate (BDH; MW 500,000)) added to each of the Multiprobe device panels. The Multiprobe device and slide were then brought into contact (with the probes and corresponding squares matching) and the arrangement carefully placed slide side underneath onto a 75 °C hot block for 1 min 50 s. It was then transferred into a light-proof slide box floating in an uncovered 37 °C water bath and hybridization allowed to proceed overnight. The Multiprobe device was then detached from the slide and the slide washed at 45 °C for 5 min in three changes of 50% formamide/1 × SSC (pH 7.0) and one change each of 1 × SSC and ST (4 × SSC + 0.05% Tween 20). Following a 35-min incubation at 37 °C in bovine serum albumin (BSA) blocking solution (3% BSA in ST), the slide was treated with three layers of fluorescein-conjugated antibodies diluted in BSA blocking solution: (1) 1:50 mouse anti-digoxin fluorescein isothiocyanate (FITC) (Sigma) plus 1:15 avidin Texas Red (Vector); (2) 1:500 rabbit anti-mouse FITC (Sigma) plus 1:3 biotin mouse anti-avidin (Vector), and (3) 1:500 goat anti-rabbit FITC (Sigma) plus 1:15 avidin Texas Red (Vector). Each antibody layer was applied directly to the slide, a parafilm coverslip overlaid and the layer incubated at 37 °C for 10 min. Following each incubation, the parafilm was removed and the slides washed 3 times in ST on a shaking platform. Finally, the slides were mounted with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in Vectashield (Vector) and the coverslips sealed in place with rubber glue. When dry, the slides were stored in a light-proof container at 4 °C until ready to view.

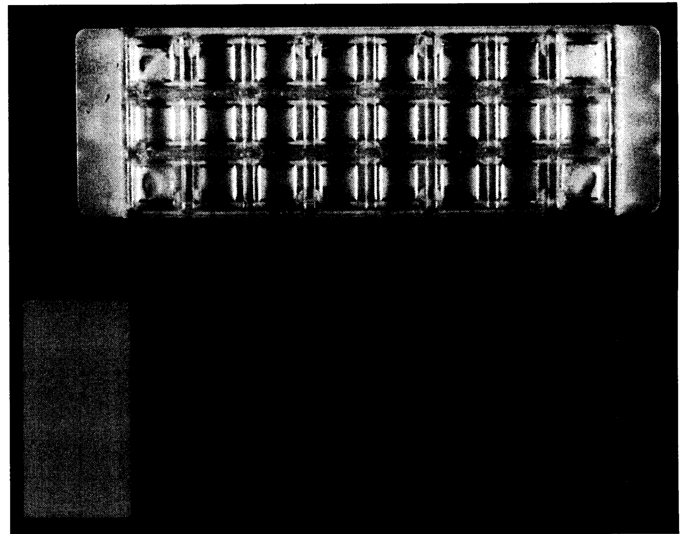

Fig. 1. Photograph of the 'Multiprobe coverslip device' (above) divided into 24 raised square platforms and the 'template microscope slide' (below) demarcated into 24 squares.

Analysis of Hybridized Samples and Interpretation of Results

The hybridized chromosome spreads were viewed using a fluorescence microscope (Olympus BX60) equipped with a Pinkel filter wheel containing DAPI, Texas Red, FITC, Dual and Triple filters. Using the Triple bandpass filter, the chromosome fluoresced light blue (DAPI) whereas the biotin-labelled p-arm probes fluoresced red (Texas Red) and the digoxigenin-labelled q-arm probes fluoresced green (FITC). Slides were scored simply by the number of telomeric signals for each metaphase. For each square, 4 hybridized metaphases were analysed and scored. Each slide took 1–1.5 h to scan.

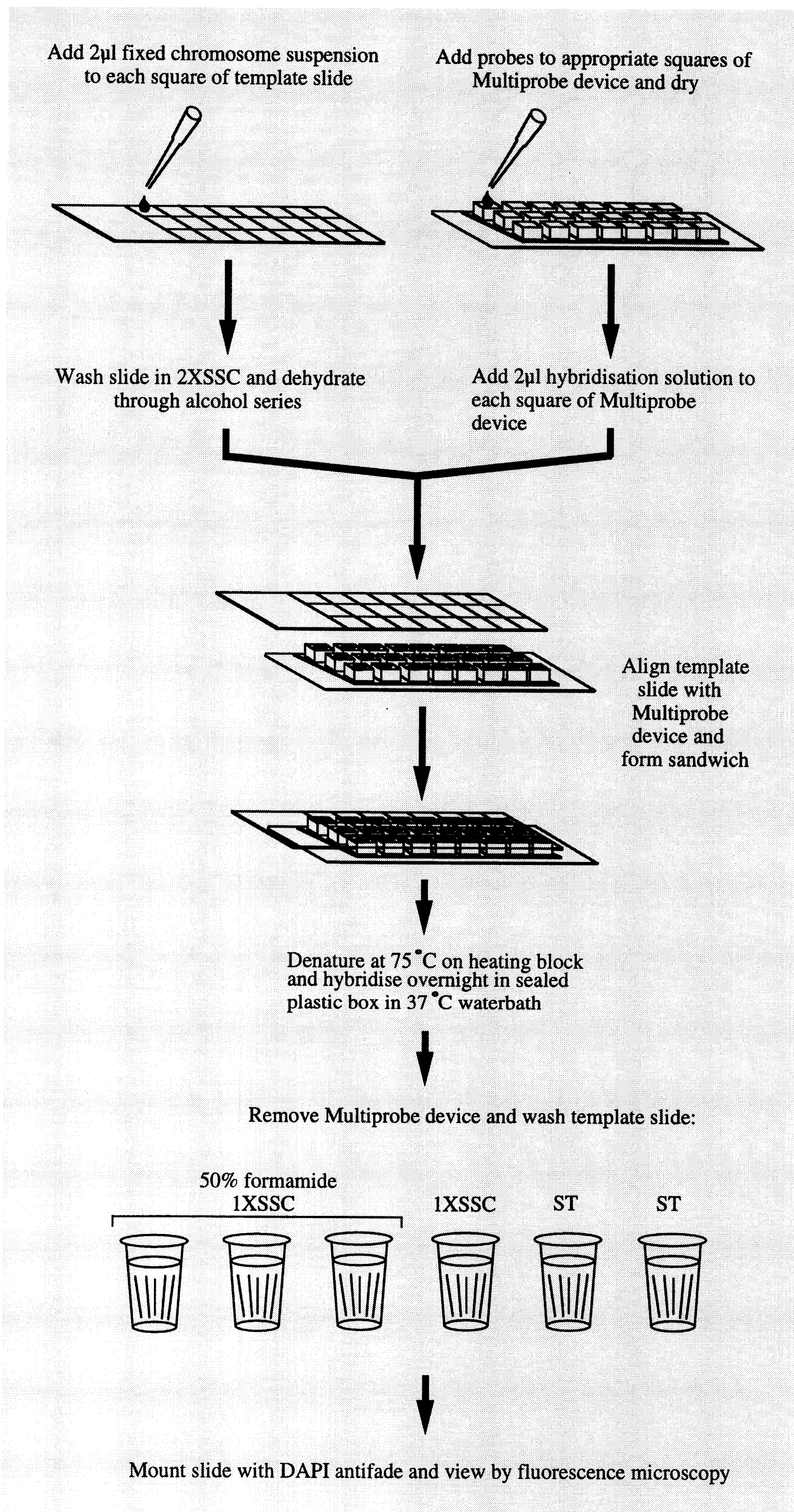

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Fig. 2. Schematic showing the overall strategy of the innovative multiple hybridization FISH technique.

Fig. 3. Multiprobe FISH results showing a complete panel of probes hybridized against a single template microscope slide prepared from a normal male fixed chromosome suspension ('1' to '22' and 'XY') and the 'X' Square result ('XX') from a complete panel of probes hybridized against a single template microscope slide prepared from a normal female fixed chromosome suspension. For each square, a single hybridizing metaphase is shown with the p-arm telomere probes signals appearing red and the q-arm probes, green.

1 2 3 4
5 6 7 8
9 10 11 12
13 14 15 16
17 18 19 20
21 22 XX XY

3

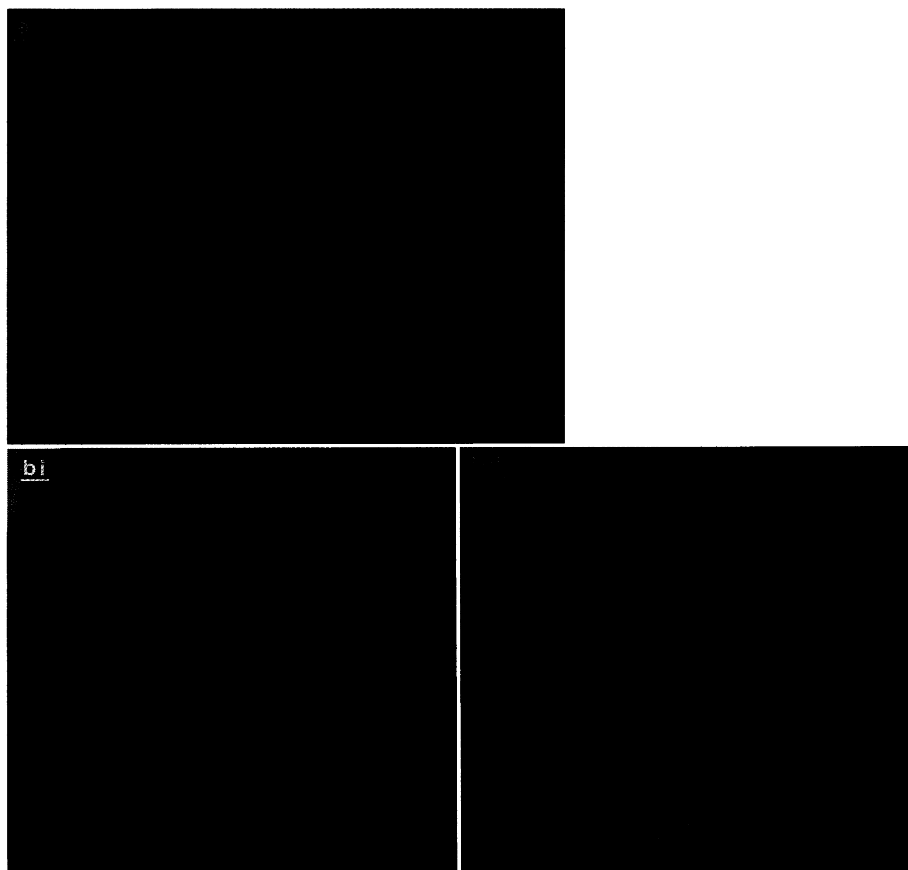

Fig. 4. Multiprobe FISH results showing (**a**) the '1' Square result from an IMR male known to have a de novo deletion of the subtelomeric region of chromosome 1p; (**bi**) and (**bii**) respectively, the '9' and '22' Square results from the IMR female with a de novo unbalanced chromosome derived from a translocation involving the terminal regions of 9q and 22q resulting in a trisomy for subtelomeric 9q and a subtelomeric deletion of 22q. For each square, a single hybridizing metaphase is shown with the derivative chromosome arrowed and the p-arm telomere probe signals appearing red and the q-arm probes, green.

Results

The reliability of the technique was confirmed by a complete set of results being obtained in separate hybridizations performed on 20 fixed chromosome suspensions from different individuals. All of the tested samples had at least 80–90% good quality metaphases in the hybridized area which gave interpretable hybridization signals (data not shown). The FISH results from the full complement of telomere probes hybridized against a single template microscope slide prepared from lymphoblastoid fixed chromosome suspensions of the normal male are shown in panels '1' to '22' and 'XY' of figure 3. Panel 'XX' shows the pseudoautosomal XpYp and XqYq probes hybridizing to the two X chromosome telomeres of the normal female. The FISH results for the male patient with IMR confirmed the subtelomeric deletion of one of the chromosome 1p homologues (fig. 4a). No other subtelomeric deletion or triplication events were detected in this individual. Similarly, the IMR female revealed the expected 9q triplication event translocated on to the deleted chromosome 22q and no other abnormality (fig. 4bi, bii).

Discussion

Currently, no useful molecular diagnostic test exists for the routine detection of submicroscopic rearrangements in patients who are suspected to have a chromosomal anomaly, but for whom there are no clues regarding the chromosomal origin. The advent of a complete set of telomere-specific probes has allowed us to develop an innovative FISH based diagnostic technique which is specific for the detection of all deletion, triplication and balanced translocation events involving subtelomeric regions [8]. Using only a single microscope slide per patient, our technique permits the simultaneous analysis of every chromosome for deletion, triplication and balanced translocation events.

Recently, Schröck et al. [4] and Speicher et al. [5] have reported the multicolour labelling of a complete set of WCPP and their hybridization to single chromosome spreads on single microscope slides. This technology provides a possible alternative strategy to the one which we have presented for the screening of subtelomeric regions. Instead of the multicolour labelling of specific chromo-

somes, it is conceivable that telomere-specific clones could be labelled so that each telomere is detected in a different colour. Using this approach, the multiple hybridization necessary could be performed not only on a single microscope slide, but also on a single metaphase spread. There are currently two main difficulties with the multicolour labelling of telomere-specific clones: (1) the majority of published telomere-specific clones are cosmids and the technique is difficult to develop for cosmid labelling and detection, and (2) interpretation of the results requires additional specialized microscope and computer equipment, currently inaccessible to diagnostic laboratories. By contrast, the FISH technique presented here has the advantage that the analysis of each square is uncomplicated and cytogenetic laboratories interested in the proposed diagnostic test will require no novel or expensive equipment or materials, thus making it cost-effective and ready for immediate use.

The results from the 4 test cases presented here demonstrate the potential of the novel FISH approach in a diagnostic environment. All of these individuals were originally reported to have a normal karyotype on conventional cytogenetic analysis. For the normal male and female tested, the normal karyotypes were confirmed. For 1 IMR patient, a subtelomeric deletion of one of the chromosome 1p homologues was identifiable and no other subtelomeric rearrangements were found, indicating that the 1p deletion may represent a truncation event. Analysis of the other IMR patient confirmed a trisomy of subtelomeric 9q associated with a deletion of subtelomeric 22q as previously reported [6].

In summary, we have devised a FISH technique which exploits a unique resource of 41 telomere-specific probes and allows the simultaneous analysis of every chromosome for deletion, triplication and balanced translocation events on a single microscope slide. The result is a simple, accurate and efficient technique which is a significant complement to chromosome-specific unique sequence probe and WCPP approaches and has a broad range of applications in clinical cytogenetics. It is particularly suited to the detection of subtelomeric rearrangements in individuals with IMR, for the identification of congenital anomalies and for aneuploidies and some chromosomal abnormalities found in cancer patients. It is also suitable for the assessment of couples who experience recurrent spontaneous miscarriages. The use of the innovative multiple FISH technique in cytogenetic diagnostic laboratories is expected to alleviate many of the problems of explaining the aetiology of previously undiagnosed disorders to families with affected members and those of providing accurate genetic counselling. In addition, the identification of previously undetectable chromosomal anomalies will provide a valuable opportunity for studying the mechanisms involved in creating unbalanced karyotypes and for investigating the molecular basis of the associated phenotypes.

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