

Development of a Biological Dosimeter for Translocation Scoring Based on Two-Color Fluorescence in Situ Hybridization of Chromosome Subsets

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Recently fluorescence in situ hybridization protocols have been developed which allow the pairing of individual chromosomes using DNA-libraries from sorted human chromosomes. This approach has the particular advantage that radiation induced chromosome translocations can be easily detected, if chromosomes of distinctly different colors take part in the translocation event. To enhance the sensitivity of this approach two metaphase chromosome subsets A and B (A: chromosomes 1, 2, 4, 8, 16; B: 3, 5, 9, 10, 13) were simultaneously painted in green and red color. Counterstaining of the chromosomes with DAPI resulted in a third subset which exhibited blue fluorescence only. Green-red, green-blue and red-blue translocation chromosomes could be easily detected after irradiation of lymphocyte cultures with ^{137}Cs - γ -rays. Analyses of painted chromosomes can be combined with conventional GTG-banding analyses. This new biological dosimeter should become useful to monitor both long term effects of single irradiation events and the cumulative effects of multiple or chronic irradiation exposures. In contrast to translocation scoring based on the analysis of banded chromosomes, this new approach has the particular advantage that a rapid, automated scoring of translocations can now be envisaged.

INTRODUCTION

Scoring of chromosomal aberrations in lymphocytes of peripheral blood can serve as a sensitive biological dosimeter of radiation exposures [1]. The usefulness of a biological dosimeter in the low dose range requires that scoring can be performed easily and rapidly in large numbers of cells. For this purpose the evaluation of either micronuclei derived from acentric chromosome fragments [2-4] or of dicentric chromosomes (for review see [5]) has become particularly useful. Dicentric chromosomes can be detected easily in homogeneously stained metaphases and procedures for the automated scoring of dicentrics have been developed [6, 7]. False positives and false negatives, however, impair the accuracy of such analyses particularly at the low dose range. In particular, the distinction between true dicentrics and composites of two normal chromosomes touching or overlapping end to end can be difficult. A more comprehensive analysis of structural chromosome aberrations performed on banded chromosome complements is time consuming and requires skilled personnel. A fully automated evaluation of banded

chromosome complements for structural chromosome aberrations has not been possible so far [8].

The formation of micronuclei or dicentrics may lead to cell death. Accordingly, a biological dosimeter based on such aberrations is useful for the detection of acute irradiation effects. Its value, however, is limited in cases where long term biological dosimetry is needed. Such a dosimeter can be based on the detection of reciprocal translocations in irradiated cell populations. By and large cells, such as stem cells of the red bone marrow, with reciprocal translocations would retain the same proliferative potential as normal stem cells. Accordingly, the progeny of red bone marrow stem cells with reciprocal translocations can be detected in the peripheral blood with undiminished frequency even many years after such an event has taken place [9]. In rare cases a specific genetic imbalance may lead to a growth advantage of the affected cell and finally give rise to a tumor cell population [10].

Fluorescence in situ hybridization has become a suitable new tool for the rapid detection of structural chromosome aberrations in metaphase spreads obtained from irradiated cell populations [11, 12]. In particular, "painting" of entire human chromosomes based on chromosomal in situ suppression (CISS-) hybridization protocols [13, 14] in combination with digital image analysis can be applied for the rapid evaluation of translocation chromosomes composed of differently colored chromosome segments [15-20]. Using this approach, we have evaluated chromosome translocations in patients who had received an intravascular injection of Thorotrast more than forty years ago [17]. This X-ray contrast medium contains $^{232}\text{Thorium}$ which decays mainly under emission of alpha-particles. The percentage of translocations was found to be significantly higher in exposed patients than in controls.

In the studies mentioned above, a single chromosome type, e.g. chromosome 1, occasionally two chromosome types, e.g. chromosome 1 and 2, were painted in each cell. In the present study we have painted two subsets, each comprising five chromosome pairs, simultaneously in green and red color. In this way, the number of translocations which can be simultaneously scored in each metaphase spread can be increased. It is our aim to develop a new, sensitive biological dosimeter able to detect the long term effects of single radiation exposures, as well as the cumulative effects of repeated or chronic irradiation at the low dose range.

MATERIALS AND METHODS

Cell Material

10 ml of blood was obtained from a female donor (46, XX) and irradiated at room temperature with 8 Gy of $^{137}\text{Cs-}\gamma$ -rays (662 keV). Lymphocytes were isolated, stimulated with phytohemagglutinin (PHA) to divide and cultured for 72 hours using standard techniques [21]. Colcemid arrested metaphase spreads were obtained after hypotonic treatment (0.075 M KCl) and fixation with methanol/acetic acid (3:1, vv). For control experiments 10 ml of blood was obtained from a male donor (46, XY) and cultivated as described above.

Pretreatment of slides

Prior to CISS-hybridization experiments slides were pretreated according to the following standard protocol with modifications described below. Slides were digested with RNase A (100 $\mu\text{g}/\text{ml}$ $2\times\text{SSC}$) for 1 h at 37°C , washed three times, 5 min. each, in $2\times\text{SSC}$, digested with pepsin (50 mg/ml in 0.01 M HCl) for 10 min. at 37°C , washed 2×5 min. in phosphate buffered saline (PBS) and 5 min. in PBS containing 50 mM MgCl_2 and then postfixed for 10 min. at room temperature in 1% formaldehyde (Merck) in PBS containing 50 mM MgCl_2 . After another washing step in PBS slides were dehydrated in 70%, 90% and 100% ethanol.

In some experiments chromosomes were banded prior to in situ hybridization using a standard protocol for GTG-banding [22] and photographed with Agfa ortho 16 DIN black and white negative films. The oil was removed by xylene and xylene/ethanol (1:1), 5 min. each. Slides were then destained in methanol/acetic acid (3:1, vv), 2×5 min. After an ethanol series with descending concentrations (100%, 90% and 70% ethanol, 3 min. each) and 5 min. washing in PBS slides were postfixed for 10 min. at room temperature in PBS containing 3.7% formaldehyde [23]. After washing steps in PBS and in $2\times\text{SSC}$ slides were digested with RNase A and pepsin and postfixed in 1% formaldehyde as described above.

DNA libraries and CISS-hybridization

Bacteriophage libraries from sorted human chromosomes 3 (LA03NS02), 5 (LA05NS01), 9 (LL09NS01) were obtained from the American Type Culture Collection. Plasmid libraries from sorted human chromosomes 1, 2, 4, 8, 10, 13, 16 were kindly provided by Dr. Joe Gray [24]. Amplification of these libraries and isolation of bacteriophage and plasmid DNA were carried out according to standard protocols [25]. Library DNAs from several sorted chromosomes were mixed to generate two probe sets A and B. Probe set A contained the library DNAs for chromosomes 1, 2, 4, 8 and 16, probe set B contained the DNAs for chromosome 3, 5, 9, 10 and 13. Optimum DNA amounts for each chromosome were determined empirically in order to achieve painting with a roughly similar intensity on all chromosomes of a given subset. For example, in our present experiments one μg DNA of probe set A contained 170 ng + 240 ng + 110 ng + 240 ng + 240 ng of plasmid DNA from chromosome libraries 1, 2, 4, 8, 16, respectively. One μg DNA of probe set B contained 240 ng bacteriophage DNA each from the chromosome libraries 3, 5, 9, 120 ng plasmid DNA from chromosome library 10 and 160 ng plasmid DNA from chromosome library 13. These DNA amounts should be taken as a hint and may have to be modified for the particular needs of other laboratories. Nicktranslation of the probe sets was carried out either with biotin-11-dUTP (Sigma) or digoxigenin-11-dUTP (Boehringer Mannheim) using standard protocols [26]. 10 μl of hybridization mixture contained 0.5 to 1 μg of each probe set, 25 μg human Cot 1-DNA (BRL) and 25 μg sonicated salmon sperm DNA (Sigma). CISS-hybridization was carried out as described [13, 26]. Biotin-labeled probes were detected using avidin conjugated to rhodamine isothiocyanate (XRITC) (Vector Laboratories). Signals were amplified once [11] using biotin-conjugated antibodies against avidin (Vector Laboratories) followed by another rhodamine-avidin step. Digoxigenin-labeled probes were detected by indirect immunofluorescence using a mouse monoclonal antibody against digoxigenin (Boehringer Mannheim) and an anti mouse IgG

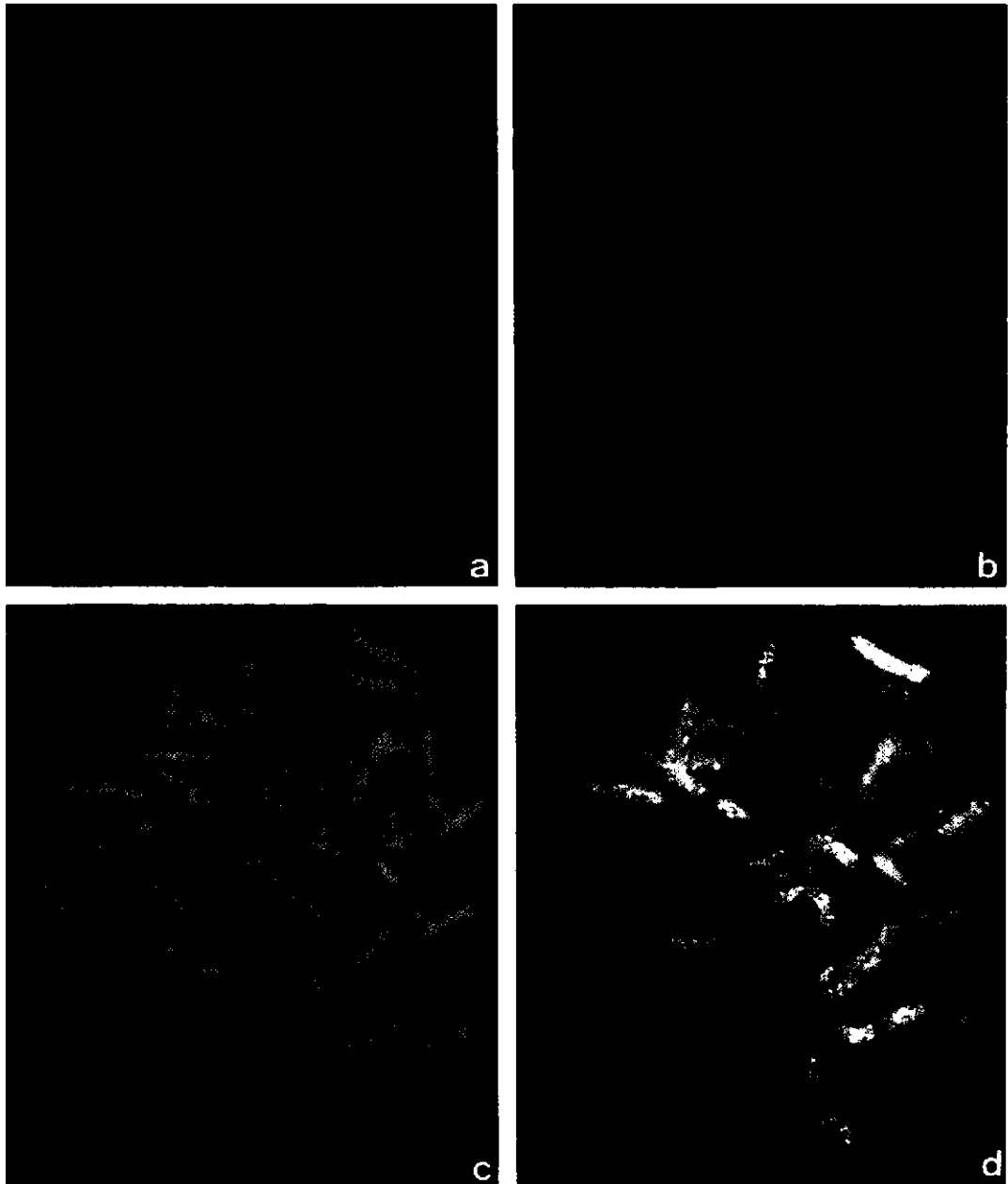
conjugated with fluorescein isothiocyanate (FITC) made in sheep (Sigma). Signal amplification was obtained with anti-sheep IgG conjugated with FITC made in donkey (Sigma). Cells were counterstained with 0.2 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) and mounted in fluorescence antifading buffer [27]. Cells were viewed with a Zeiss photomicroscope Axiophot equipped for epifluorescence using a Plan-Neofluar 100 \times /1.3 oil objective. Filter combinations used were as follows: FITC: BP 450–490, FT 510, LP 515–565 or LP 520; XRITC: BP 546, FT 580, LP 590; DAPI: BP 365, FT 395, LP 397. For microphotographs with double and triple exposures a double band pass filter for the simultaneous FITC- and rhodamine-detection (Carl Zeiss, Oberkochen, Germany) was used. Microphotographs were taken on Agfachrome 1000 ASA color slide films.

RESULTS

Two color CISS-hybridization with two probe sets A and B was applied to metaphase spreads prepared from normal human lymphocyte cultures and cultures irradiated with 8 Gy of ^{137}Cs - γ -rays. Probe set A painted the human chromosomes 1, 2, 4, 8 and 16, probe set B chromosomes 3, 5, 9, 10 and 13. After detection of the hybridized chromosomes with the fluorochromes FITC or rhodamine a green and a red chromosome subset could be easily distinguished (Fig. 1a, b). Counterstaining of the whole chromosome spread with DAPI (Fig. 1c) results in a third chromosome subset exhibiting blue fluorescence only (compare Fig. 1c with 1d). In the following this subset is briefly referred to as blue chromosome subset although the red and green painted chromosomes were simultaneously stained with the blue DAPI fluorochrome. Accordingly, any translocation occurring between chromosomes of differently colored subsets could easily be scored in the irradiated cell sample, including green-red, green-blue, red-blue translocation chromosomes, even when the amount of translocated chromosome material was small (see Fig. 2a-f, k-p, for examples). GTG-banding was performed prior to CISS-hybridization and allowed conventional chromosome banding analyses in addition to the analyses of painted chromosomes (Fig. 2m, p, s, v). While the unequivocal identification of aberrant chromosomes was often difficult or even impossible when based solely on chromosome banding, the contribution of chromosome material from each of the three distinctly colored

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- Fig. 1.** Peripheral human lymphocyte metaphase spread (46, XY) after two-color CISS-hybridization with probe set A (biotin-labeled DNA from human chromosome libraries 1, 2, 4, 8, 16) and probe set B (digoxigenin-labeled DNA from human chromosome libraries 3, 5, 9, 10, 13).
- a: Chromosome pairs 3, 5, 9, 10 and 13 painted with fluorescein isothiocyanate (FITC, green fluorescence).
 - b: Chromosomes 1, 2, 4, 8 and 16 painted with rhodamine isothiocyanate (red fluorescence).
 - c: Metaphase after counterstaining with DAPI (blue fluorescence).
 - d: Same metaphase after triple exposure. Note the three subsets of chromosomes painted green, red or blue only.

chromosome subsets to aberrant chromosomes, such as translocation chromosomes (see above), ring chromosomes (Fig. 2g, h, t-v), chromosome fragments (Fig. 2q-s), as well as to micronuclei (Fig. 2i, j) could be unequivocally determined.



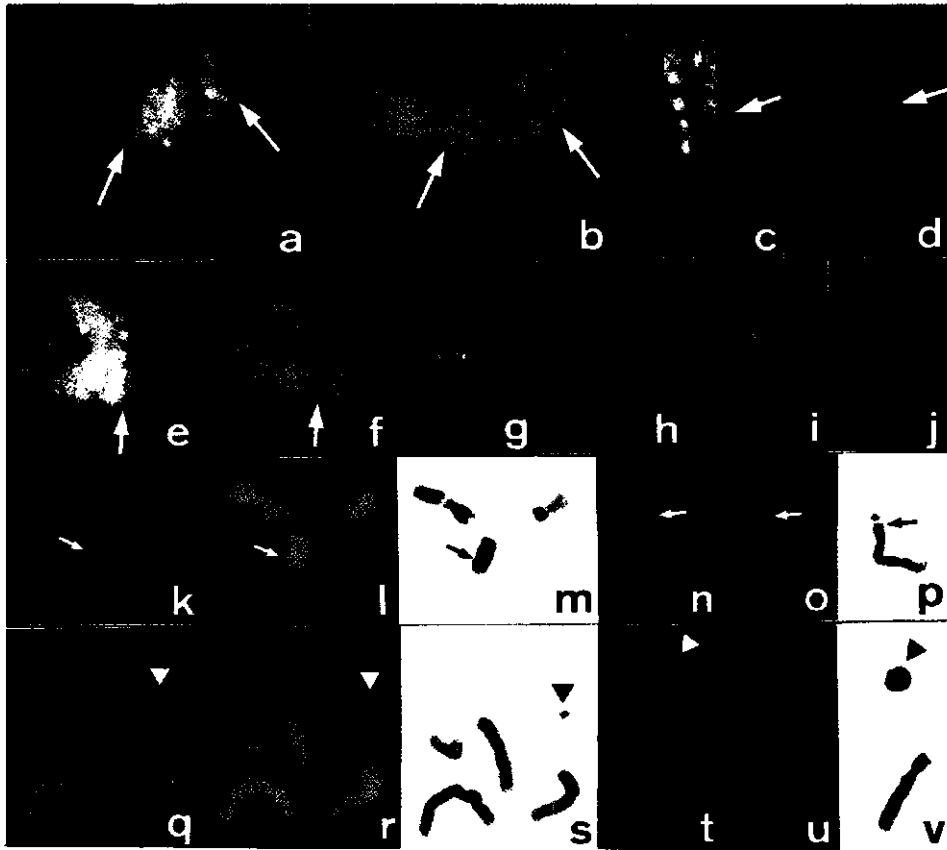


Fig. 2. Metaphase chromosome spreads with multiple chromosome aberrations obtained from a peripheral human lymphocyte culture (46, XX) irradiated with 8 Gy of ^{137}Cs - γ -rays after two-color CISS-hybridization with DNA probe set A (human chromosome libraries 1, 2, 4, 8, 16) and probe set B (human chromosome libraries 3, 5, 9, 10, 13). a, b, g-j: probe set A labeled with digoxigenin and detected with FITC-conjugated antibodies (green fluorescence); probe set B labeled with biotin and detected with rhodamine-conjugated avidin (red fluorescence); c-f, k-v: probe set A labeled with biotin (red fluorescence); probe set B labeled with digoxigenin (green fluorescence).

a, b) Translocation chromosome with green painted chromosome material (a) counterstained with DAPI (b). Arrows point to the breakpoint regions.

c, d) Endoreduplicated translocation chromosome detected with probe set A (red) and probe set B (green) (c) and counterstained with DAPI (d). Arrow points to the breakpoint region.

e, f) Partial metaphase spread shows two painted chromosomes and one non-painted chromosome (e) all counterstained with DAPI (f). An acrocentric chromosome (#13) is painted entirely green. Part of a submetacentric translocation chromosome is painted red, while the other part is exclusively stained with DAPI. Arrow points to the breakpoint region. A small metacentric chromosome (left) is stained with DAPI. Note that very small translocations may not be distinguished from background. For example, two red fluorescent dots on this chromosome are considered as background dots.

g, h) Ringchromosome entirely painted with probe set B (red fluorescence; g), counterstained with DAPI (h).

DISCUSSION

This study demonstrates that two-color fluorescence in situ hybridization of chromosomal subsets can be used to develop a biological dosimeter based on translocation scoring. Two chromosomal subsets (combining chromosomes 1, 2, 4, 8, 16 and 3, 5, 9, 10, 13, respectively) were detected with green and red fluorochromes in different combinations. A third subset comprising the non-hybridized chromosomes exhibited blue fluorescence only after counterstaining with DAPI. With this approach any translocation of at least a few megabase size between chromosomes of different color can be easily detected. Painting of chromosome subsets can be combined with conventional chromosome banding analysis (e.g. [23, 26]). Translocation scoring based on a distinct change of color along an individual chromosome should provide a new and particularly useful possibility for automation. Sensitive color CCD-cameras are presently being developed and may be used to digitize images from multicolored chromosome spreads for further analyses. It can be expected that such an approach will also help to facilitate the automated image analysis of overlapping chromosomes painted in different colors. Additional labelling of centromeric and telomeric chromosomal subregions can be used to distinguish between true di- or multicentric chromosomes and composite chromosomes (false positives). In conclusion, multicolor painting of chromosome subsets should result in a long term biological dosimeter with considerably improved sensitivity as compared to the routine evaluation of dicentric chromosomes.

The general applicability of the biological dosimeter described in this report is presently limited by the requirements of multicolor chromosome painting in routine cytogenetic laboratories. Limitations due to the difficulties of probe generation, labeling and detection protocols, however, will likely be overcome in the near future when painting probes directly conjugated with appropriate fluorochromes become commercially available. In addition, digital fluoresc-

i, j) Micronucleus derived from chromosome material detected with both probe sets A and B (i), counterstained with DAPI (j).

k-m) Partial metaphase spread shows two normal chromosomes 11 and 17 and an aberrant chromosome after CISS-hybridization (k), counterstaining with DAPI (l) and GTG-banding (m). The aberrant chromosome shows a small translocation painted green with probe set B (arrow). The rest of this chromosome is stained with DAPI. The two normal chromosomes are also not painted.

n-p) Translocation chromosome composed of two chromosome subregions painted with probe sets A (n) and B (o) after GTG-banding (p). Arrows point to the breakpoint region.

q-s) Partial metaphase spread shows four chromosomes and a small chromosome fragment (arrowhead) after two-color CISS-hybridization (q), counterstaining with DAPI (r) and GTG-banding (s). Two normal chromosome 9 homologs and the fragment are painted green, while a normal chromosome 4 is painted red. A normal chromosome 19 is exclusively stained with DAPI.

t-v) Partial metaphase spread shows two chromosomes with GTG-banding (v), a green painted ring chromosome (t) and a red painted chromosome 4 (u).

ence microscopy will help to increase the number of useful fluorochromes. For example, even fluorochromes emitting in the infrared can be included. In addition, painting probes may be labeled with two fluorochromes simultaneously. In this way chromosomes with colocalization of two fluorochromes can be easily distinguished from other chromosomes which show fluorescence from a single fluorochrome only [28]. When designing improved versions of a biological dosimeter based on multicolor chromosome painting, one has to weigh the potential benefit of more sophisticated painting protocols which also require more sophisticated hardware and software for automated evaluation against the practicability of such protocols. Our goal is to increase the sensitivity of the translocation scoring to an extent which is useful for dosimetry in the low-dose range. This approach could also be applied to study the effects of ageing on the frequency of interchromosomal rearrangements in various cell types of a normal population.

The approach described in this paper should also be useful to define the composition of marker chromosomes in clinical and tumor cytogenetics which cannot be analyzed by classical banding procedures alone. Using multicolor CISS-hybridization with probes for several chromosomal subsets the chromosomal origin of a marker chromosome can be narrowed down rapidly to a few candidate chromosomes. Additional multicolor CISS-hybridization experiments with individual chromosome libraries and chromosome band specific DNA probes can then be defined to pinpoint the exact origin of the marker chromosome.

As compared to translocation scoring by conventional banding analyses the requirements for chromosome spreading are less demanding in multicolor chromosome painting experiments. In the latter approach translocations can be easily detected even in metaphase spreads with poor spreading. Furthermore, overlapping chromosomes of different color can be more easily segmented with appropriate filtering (our unpublished data). Still, the requirement of mitotic cells for translocation scoring provides a major obstacle for the application of a biological dosimeter, for example in cases of local irradiation of tissues from which metaphase spreads cannot easily be prepared in sufficient numbers. Therefore, it would be highly desirable, if a biological dosimeter could be developed for chromosome aberration scoring directly in cell nuclei of irradiated tissues. In contrast to the painting of individual chromosomes which has been supplied successfully for the scoring of chromosome translocations in the interphase nucleus [15], [16], nuclear painting patterns derived from the painting of chromosomal subsets as described in this paper are not suitable for such interphase cytogenetic analyses. The sensitivity and accuracy with which chromosome translocations can be detected in interphase nuclei after multicolor painting of individual chromosome domains and the potential of such an approach for automated analyses provides an important field for future investigations. Region specific probes are generally more useful for interphase cytogenetics than whole chromosome paint libraries. The number of such probes useful for the interphase detection of specific numerical or structural chromosome aberrations is rapidly increasing. Using such probes, it may become possible to detect specific subpopulations of interphase cells containing irradiation induced preneoplastic or neoplastic chromosome aberrations, for example in the bone marrow of an irradiated patient.

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