

Detection of Chromosome Aberrations by FISH as a Function of Cell Division Cycle (Harlequin-FISH)

BioTechniques 26:532-534 (March 1999)

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ABSTRACT

Chromosome aberrations are a sensitive indicator of genetic change, and the measurement of chromosome aberration frequency in peripheral blood lymphocytes is often used as a biological dosimeter of exposure (1,4). The length of time that cells are maintained in culture before cytogenetic analysis is probably the most important *in vitro* factor that influences both the frequency and types of aberrations that are seen following exposure to mutagens. Therefore, for accurate cytogenetic measurements of genetic damage, cells must be analyzed in their first mitosis following exposure. As cells progress through subsequent mitotic division cycles, cells with unstable types of aberrations, e.g., dicentrics and acentric fragments, are eliminated (1,3,4). Even the use of synchronized populations of cells does not guarantee that all cells analyzed will be in their first division following treatment. Small variations in growth rate after irradiation can lead to large variations in the proportion of cells that are in their first vs. a subsequent mitosis. For example, 48 h after G₀ lymphocytes are stimulated to enter the cell cycle (the standard sampling time for cytogenetic analysis), up to 50% of the cells in mitosis can be in their second division cycle (10). While there are methods available to distinguish cells in different di-

vision cycles (see Introduction), they are not easily adapted for use with standard fluorescence *in situ* hybridization (FISH) procedures. The goal of this study was to develop a simple approach to detect aberrations by FISH whereby cells in different division cycles could be distinguished.

INTRODUCTION

The standard method used to distinguish cells in different division cycles is harlequin chromosome staining (5,9,10). It involves culturing cells in 5-bromo-2'-deoxyuridine (BrdUrd) and then detecting the BrdUrd-containing DNA in chromosomes using Hoechst 33258 fluorescent dye. The BrdUrd-containing DNA quenches the Hoechst dye, and the amount of quenching is related to the relative amount of BrdUrd incorporated into the chromosomes. In cells that have gone through two division cycles in the presence of BrdUrd, one sister chromatid is doubly labeled with BrdUrd, while the other is singly labeled. This difference is detected by Hoechst fluorescence. Sister chromatids of cells in their first division cycle show equal fluorescence, while those in cells that have gone through more than two division cycles show patterns consistent with >50% of the genome being doubly labeled with BrdUrd. Modification of the harlequin staining, by combining the fluorescent dye with a Giemsa stain (fluorescence-plus-Giemsa), allows for a permanently stained preparation (9).

However, one limitation of the harlequin staining procedure is that it only allows for the detection of unstable types of aberrations such as dicentrics

and acentric fragments, wherein the abnormal structure of the chromosome is easily detected. Most investigators now use fluorescence *in situ* hybridization (FISH) procedures to detect both unstable and stable types of aberrations including translocations and inversions (2,3,11). Unfortunately, FISH cannot be easily combined with harlequin staining to allow scoring of both types of aberrations as a function of the cell division cycle. Boei and colleagues (2,3) reported using FISH and harlequin staining either combined or sequentially to determine aberration frequency as a function of cell division cycle. However, the resolution of individual chromosomes when both procedures are combined is poor, and applying the two procedures in sequence is quite tedious because it involves recording the location of all metaphases containing aberrations and reexamining them later with harlequin staining.

Goodwin and colleagues (8) recently described a chromosome orientation and direction FISH (COD-FISH) technique to identify specific gene orientation. The procedure involves culturing cells for one division cycle in BrdUrd to singly label sister chromatids. Mitotic preparations are then stained with Hoechst dye and exposed to UV radiation, which leads to the selective production of DNA breaks at sites of BrdUrd incorporation. These samples are subsequently treated with exonucleases to digest the BrdUrd-containing strand and eliminate its ability to hybridize with any labeled genetic probe. Goodwin and colleagues developed this procedure to determine the orientation of a specific gene on a particular chromosome. We adapted the technique to

use with whole-chromosome probes and thereby created a simple, single-step approach to combining harlequin staining with FISH.

MATERIALS AND METHODS

The human B-lymphoblastoid cell line, TK6 (6,7) was used to develop the harlequin-FISH procedures. TK6 cells have a cell-doubling time of about 24 h.

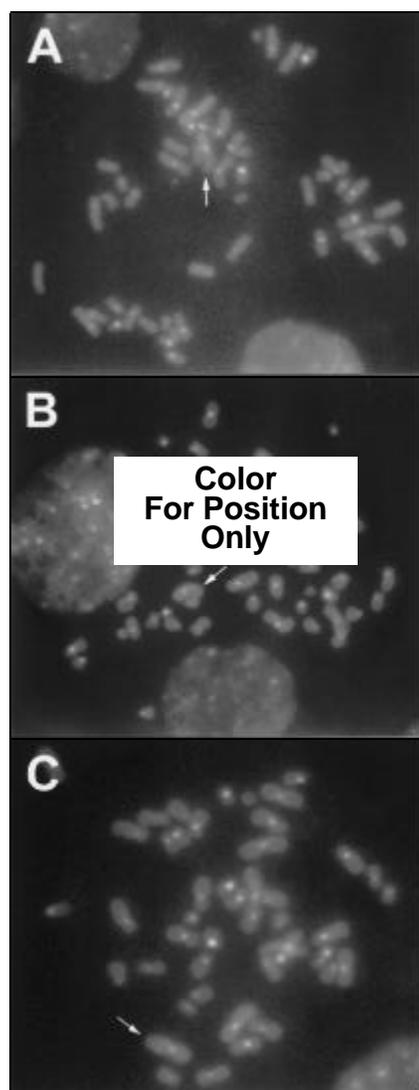


Figure 1. Harlequin-FISH staining of TK6 metaphase preparations. TK6-metaphase cells were treated for harlequin FISH and hybridized with a pan-centromeric probe and a chromosome 2 probe (Oncor). (A) A first-division metaphase with a dicentric chromosome (arrow). (B) A second-division cell with a reciprocal translocation involving chromosome 2 (arrows). (C) A second-division cell with a sister chromatid exchange on chromosome 2 (arrow).

Asynchronous cultures of cells were exposed to ^{137}Cs - γ rays to induce chromosome aberrations. TK6 cells were cultured in the presence of 20 μM BrdUrd (Sigma, St. Louis, MO, USA) for 40 h following irradiation. Cells were protected from light to prevent any BrdUrd photolysis. Two hours before cell harvesting, 0.2 μM COLCEMIDTM (Life Technologies, Gaithersburg, MD, USA) was added. The cells were then harvested by standard technique (8). Briefly, cells were spun out of media and incubated in a 0.075 M hypotonic KCl solution for 20 min at 37°C. After hypotonic treatment, the cells were washed twice with methanol/acetic acid (at 3:1) and then dropped onto slides.

Slides were typically allowed to age at room temperature (RT) for 24–72 h before processing, and they could be stored for up to 1 month in the dark at RT before further processing. Slides were first processed by incubation for 15 min in 5 $\mu\text{g}/\text{mL}$ Hoechst 33258 dye (Sigma) in Sorenson's buffer (0.03 M KH_2PO_4 , 0.03 M Na_2HPO_4). After washing off the Hoechst dye and allowing the slides to air dry, they were mounted in a few drops of Sorenson's buffer and exposed to long-wave UV light (356 nm) for 30 min on a 55°C slide warmer. The exposure to UV light preferentially induces DNA single-stranded breaks in the BrdUrd-containing DNA (8). These breaks are suitable substrates for exonucleases. Air-dried slides were treated with 50 μL of Exonuclease III (3 U/ μL ; Promega, Madison, WI, USA) for 15 min at RT. This treatment is three times longer than what Goodwin and colleagues (8) reported, but, in our experience, this longer exonuclease treatment gives clearer, more obvious differential staining of BrdUrd-substituted chromatids. After a final wash in water, the slides are processed with standard FISH procedures (11) and hybridized with whole-chromosome probes.

RESULTS AND DISCUSSION

Examples of harlequin-FISH staining are shown in Figure 1. In this sample, chromosomes were stained with both a directly labeled probe for human

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chromosome 2 and a pan-centromeric probe (Oncor, Gaithersburg, MD, USA). Directly labeled probes gave a more reliable signal than biotin- or digoxigenin-labeled probes. For cells in their first division cycle, both sister chromatids show the same level of intensity (Figure 1A) because each contains an unsubstituted DNA strand. In second-division cells (Figure 1, B and C), the very poor fluorescence intensity on the doubly BrdUrd-substituted chromatid is easily distinguished from the singly substituted chromatid. Exonuclease treatment effectively eliminated the substituted strands, thereby eliminating any chromosome 2 hybridization to the doubly substituted chromatid. The same pattern can be seen for the centromeres on chromosome 2 and on the other chromosomes as well.

Exonuclease treatment effectively eliminated the centromere hybridization signal on the doubly substituted chromatid. The intensity of the 4',6-diamidino-2-phenylindole (DAPI) counterstain is also reduced on the doubly substituted strand. Fluorescence intensity differences between singly and doubly substituted strands were more obvious when propidium iodide was used as a counterstain. Normal variations in centromere signal and counterstain intensity made the use of these fluorescence patterns a less reliable marker of the cell division cycle than the whole-chromosome probe.

The harlequin-FISH procedures did not adversely affect our ability to detect chromosome aberrations (Figure 1). Dicentrics and translocations were easily detected. In addition, the harlequin-FISH procedures allowed for the analysis of aberrations in both first- (Figure 1A) and second-division cells (Figure 1B) on the same slide. This method also allows the simultaneous measurement of sister chromatid exchange (SCE) frequency involving the painted chromosome (Figure 1C). The efficiency of detecting first- and second-division cells did not seem affected by the staining procedure. We found similar frequencies of second-division cells by standard Giemsa-harlequin staining and Harlequin FISH (29% vs. 34% in one experiment, and 41% vs. 44% in another). The technique also worked equally well in peripheral blood lymphocytes cultured from whole blood.

Measuring chromosome aberration frequencies by FISH has become an important method of biological dosimetry following mutagen exposure (11). Ensuring an accurate cytogenetic determination requires that the division cycle of the cells analyzed be known. Our harlequin-FISH procedures provide a simple and reliable method to ensure accuracy. In addition, they provide a method to analyze the both structural aberrations and SCEs as a function of division cycle on the same slides.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Grant No. CA-73931.

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Received 8 September 1998; accepted 4 December 1998.

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