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METHODOLOGY

A SIMPLE PROCEDURE FOR REHYBRIDIZATION OF NUCLEI ANALYZED PREVIOUSLY BY FISH

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ABSTRACT

We present a simple procedure for interphase fluorescence *in situ* rehybridization (FISH). This procedure was used to evaluate fresh prostate tumor from needle biopsy specimens. Digoxigenin-labeled DNA probes were hybridized onto nuclei which had been previously investigated by FISH using biotin-labeled DNA probes. This method makes it possible to reanalyze the same nuclei with different centromeric DNA probes and is useful in cases where a limited number of slides are available.

Fluorescence *in situ* hybridization (FISH) provides a direct way of detecting specific DNA sequences in metaphase chromosomes and interphase nuclei. This technique has rapidly found applications in clinical and cancer research. In most FISH studies, fresh or stored fixed cells are used for hybridization experiments, and the slide is used only once (Wang-Rong *et al.*, 1995). We developed a simple procedure for FISH by which the same slide can be used for two consecutive FISH experiments with different detection systems and fluorochromes.

Tumor specimens obtained after needle biopsies from patients with prostate cancer were processed according to standard procedures (Brown *et al.*, 1994; Dierlamm *et al.*, 1996). In brief, the samples in saline solution were mechanically disaggregated and treated with hypotonic sodium citrate solution (1%) for 20 min and methanol/acetic acid (3:1) overnight. The remaining cell clumps were disaggregated with 60% acetic acid and the suspension was dropped onto cold slides which were stored at -70°C until used.

FIRST HYBRIDIZATION

Slides were pretreated with 2 x SSC, pH 7.0-7.4, for 30 min at 37°C, dehydrated in a graded ethanol series (70, 85 and 100%; 2 min each) at room temperature and air dried. An alternative pretreatment was also used as follows: the slides were washed in 70% acetic acid for 1 min at room temperature and three times in 1 x PBS for 4 min each at room temperature. Then, 100 µl DNAase-free RNAase A (Sigma; 100 µg/ml in 2 x SSC) was dropped onto a plastic coverslip, which was then placed over the pretreated slides and incubated for 45 min to 1 h at 37°C in a moist chamber with 2 x SSC, pH 7.0. After three washes for 5 min each in 2 x SSC, pH 7.0, dehydration in a graded ethanol series (70, 85 and 100%; 2 min each) and air drying, the slides were incubated in pepsin solution (Sigma; 10 mg in 100 ml 0.01 M HCl) for 5-10 min at 37°C and washed in 1 x PBS (5 min). After incubation in 4% formaldehyde/1 x PBS for 10 min at 37°C, the slides were washed in 1 x PBS (5 min), dehydrated in a graded ethanol series for 2 min each and air dried.

Cellular DNA was denatured by immersing the slides in 70% formamide/2 x SSC, pH 7.0-7.4, for 2-4 min at 72°C, followed by dehydration in a cold ethanol series (-20°C). Five microliters of hybridization mixture (30 µl Hybrisol VI Oncor and 1.5 µl centromeric probe) was denatured for 5 min at 72-74°C. After brief cooling on ice, this mixture was added to each slide, which was then covered with an 18 x 18 mm coverslip and sealed with rubber cement. Hybridization was allowed for 16-24 h in a moist chamber at 37°C. Then the slides were washed in 50% formamide and 2 x SSC for 5 min each at 37°C. After incubation in phosphate Tween 20/PBT (30% BSA, 1% Tween 20/PBS) or phosphate buffer detergent/PBD (Oncor) for 2 min at room temperature, the biotin-labeled probe was detected with fluorescein-labeled avidin at final concentration of 1:1000 wash buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% v/v Tween 20, 5% w/v non-fat dry milk powder), and the digoxigenin-labeled probe was detected with rhodamine-labeled anti-digoxigenin (Oncor). Detections were carried out for 30 and 5 min at 37°C, respectively, in a pre-warmed humidified chamber in the dark. The slides were washed in PBT or PBD (3 x 2 min) at room temperature (25-28°C). Biotin-labeled probe signals were amplified with biotinylated anti-avidin at a final concentration of 1:200 wash buffer and fluorescein-labeled avidin (Vector) and digoxigenin-labeled probe signals were amplified with rabbit anti-sheep and rhodamine-labeled anti-rabbit (Oncor). Nuclei were counterstained with 12 µl of 4′ 6-diamino-2-phenylindole dihydrochloride (DAPI) (0.1 µg/ml antifade solution/Oncor) or propydium iodide (0.6 µg/ml antifade solution/Oncor) (Figure 1A_1 and B_1).

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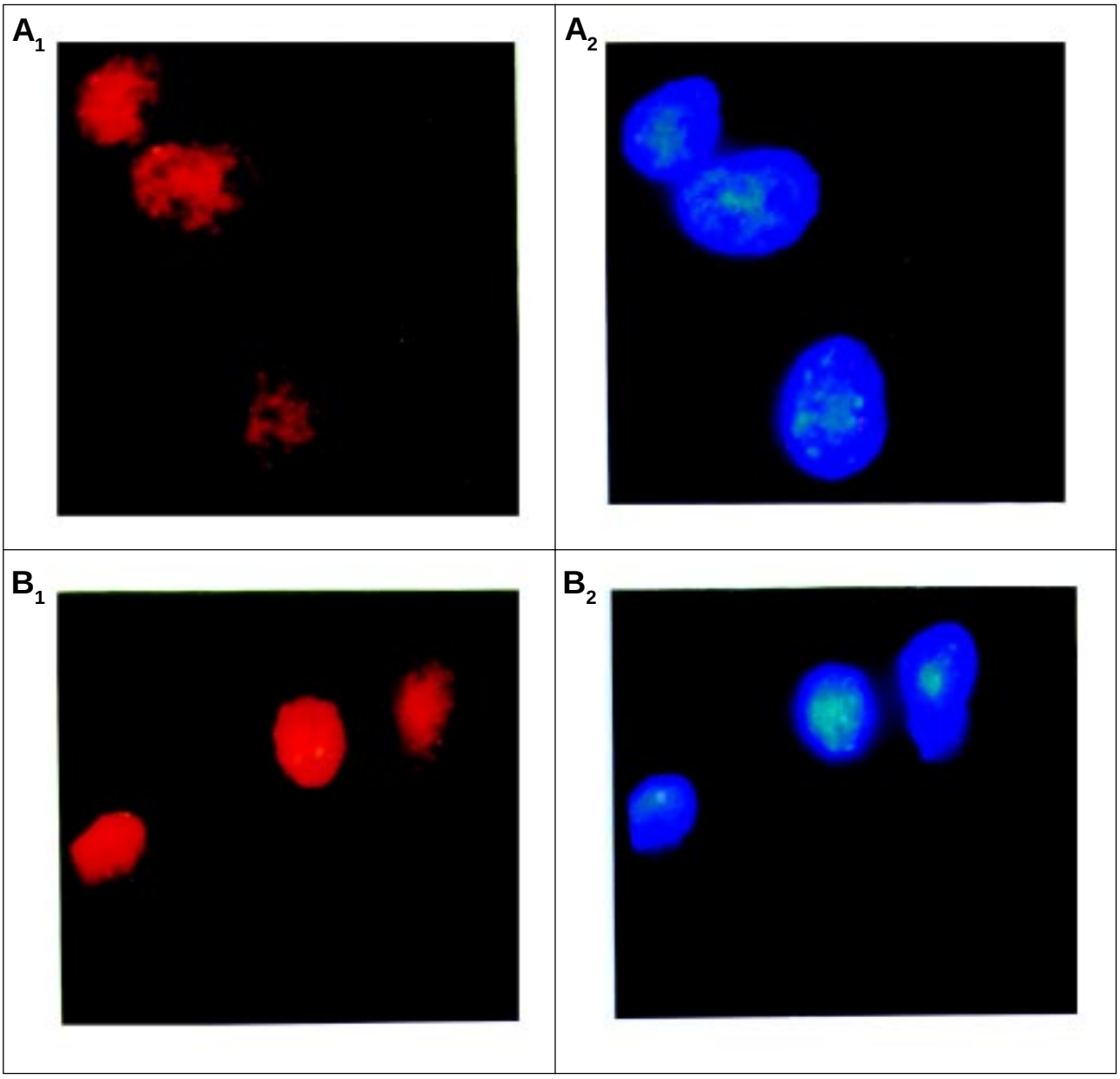

Figure 1 - Two FISH experiments were consecutively performed on the same slides of a patient with prostate cancer. A_1) First hybridization with biotinylated centromeric probe for chromosome 7 and A_2) second hybridization with digoxigenin-labeled probe for chromosome 10. B_1) First hybridization with biotinylated centromeric probe for chromosome 7 and B_2) second hybridization with digoxigenin-labeled probe for chromosome 10.

SECOND HYBRIDIZATION

The slides, after previous hybridization analysis, were conserved in the dark at -70°C for four days to one month. For the second FISH, the coverslips were gently removed, and the slides were destained in 1 x PBS, pH 7.0, for 5 min and washed in a graded ethanol series (70, 85 and 100%) for 2 min each at room temperature. Slides and probes were denatured, and hybridization was performed for 24-36 h at 37°C in a moist chamber. Post-hybridization washes, detection and amplification of the probe signal were identical to those in the procedure for the first FISH, but pretreatment was omitted (Figure 1A_2 and B_2). For each probe, signals from about 300 nuclei were evaluated by two independent observers. For each analysis, the guidelines described by Eastmond *et al.* (1995) were followed: only nonoverlapping nuclei were counted, damaged nuclei were not included, signals had to be completely separated to be scored individually, and bilobed signals that were not completely separated were counted as one. For each experiment, one slide of normal fresh skin samples was used as control. Only slides that displayed hybridization efficiency of 80% or more were scored. Signals related to probes applied in previous hybridizations persisted

with variable intensity. The morphology of nuclei was not lost during the second hybridization and the first and second hybridizations showed a high efficacy, reaching more than 85% of the nuclei.

We conclude that, with the technique described, the same slide can be used for up to two consecutive FISH experiments without loss of specificity and with variable intensity of hybridization signals.

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RESUMO

O presente trabalho descreve um procedimento simples para reibridização *in situ* fluorescente em núcleos interfásicos de amostras frescas de tumores de próstata obtidas a partir de biopsias aspirativas. Sondas α-centroméricas marcadas com digoxigenina foram hibridizadas em núcleos anteriormente avaliados com diferentes sondas marcadas com biotina. Esse método permitiu a análise da mesma célula com diferentes seqüências de DNA centroméricas, especialmente em casos com um número limitado de lâminas disponíveis.

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