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Detection of DNA Sequences in Nuclei in Suspension by in Situ Hybridization and Dual Beam Flow Cytometry

Abstract. *This report describes the fluorescence hybridization of DNA sequence probes to interphase nuclei in suspension and the quantification of bound probe by dual beam flow cytometry. Nuclear proteins were first cross-linked with dimethylsuberimidate to prevent disintegration of the nuclei during denaturation and hybridization. To demonstrate that in situ hybridization can be performed in suspension, stabilized mouse thymocyte nuclei were hybridized with a probe for mouse satellite DNA sequences. The DNA probes were labeled with 2-acetylaminofluorene. After hybridization, an indirect immunofluorescent labeling procedure was used to visualize the target sequences. With dual beam flow cytometry, both the amount of hybridized probe and the DNA content of individual nuclei were determined. Thus, the specificity of DNA hybridization can be combined with the speed and quantitative analysis provided by flow cytometry.*

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Flow cytometry is widely used for the analysis of cell nuclei and chromosomes. With this technique, the binding of fluorescent markers to individual cells or chromosomes in a heterogeneous population can be determined rapidly. After labeling with fluorescent dyes, chromatin can be analyzed quantitatively for DNA content (*1–5*), base composition (*6*), and structural proteins (*7, 8*). A logical extension of the application of flow cytometry is the detection and quantification of specific DNA sequences by fluorescence DNA hybridization techniques.

Several laboratories have developed fluorescent labeling procedures that allow the visualization of hybridized DNA sequence probes in cells fixed to slides (*9–14*). Nuclei, isolated into suspension with a procedure developed for the isolation of chromosomes (*15, 16*), fall apart rapidly under the conditions required for DNA hybridization. This can be prevented by cross-linking the nuclear proteins with dimethylsuberimidate (DMS) (*17*). Nuclei that have been treated with DMS can withstand a variety of denaturation and hybridization conditions involving incubation at high temperatures in the presence of chelating agents and high concentrations of salt. After DNA-staining with Hoechst 33258, these nuclei show normal fluorescence distributions when they are measured in a flow cytometer. The distributions consist only of a narrow peak, corresponding to the expected DNA content of the nuclei. Without DMS treatment, the peak is broad or is replaced by a debris continuum. Isolated and DMS-treated chromosomes can also be denatured by these procedures with preservation of a normal karyotype as measured in a flow cytometer (*17*).

To demonstrate in situ hybridization in suspension, DMS-treated mouse thymocyte nuclei were hybridized with a probe for mouse satellite DNA sequences. These sequences comprise approximately 10 percent of the total DNA in mouse nuclei (*18*). Total human DNA served as a control; this material shows no cross-hybridization to mouse DNA on filters or slides. Approximately 20 percent of the guanine residues in the DNA probes were chemically modified with *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF) (*9, 19*). Details of the hybridization procedure are as described (*20*). Briefly, nuclei, suspended in hybridization buffer in the presence of AAF-labeled probe, were denatured and incubated at the hybridization temperature overnight. After hybridization, the bound probe was visualized with a rabbit anti-AAF antibody (*21*) and a goat–anti-rabbit immunoglobulin conjugated to rhodamine (TRITC). Hoechst 33258, a DNA-specif-

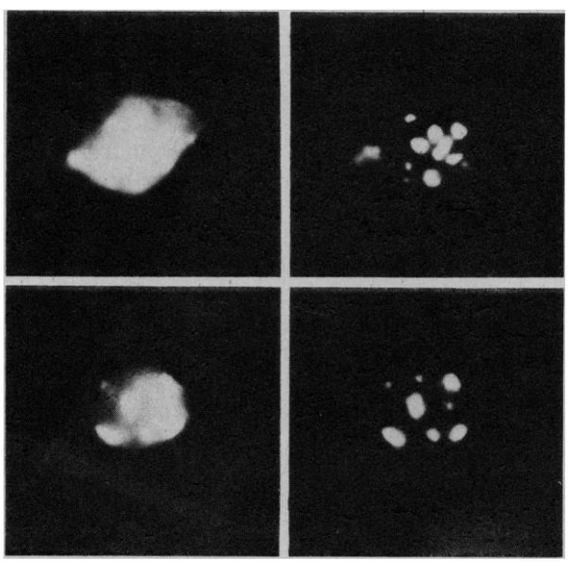

Fig. 1. Hybridization of mouse satellite DNA to mouse thymocyte nuclei in suspension. After denaturation, hybridization, and immunofluorescent labeling in suspension (*20*), a portion of the nuclei was spun onto microscope slides in a cytocentrifuge (Shandon, Cheshire, England), with the following protocol. Fetal calf serum (50 μl) was first spun onto the glass slide (10*g*, 20 seconds), followed by 250 μl nuclei suspension (300*g*, 3 minutes), and 100 μl 96 percent ethanol (300*g*, 1 minute). Microscopic observation and photography were as described (*9*). Exposure times were 15 to 30 seconds for Hoechst fluorescence and 2.5 to 3.0 minutes for rhodamine fluorescence. The two photographs on the left show examples of the blue Hoechst 33258 fluorescence, indicating the more intensely staining, A-T–rich heterochromatin areas in the nuclei. The panels on the right show the red rhodamine (TRITC) fluorescence that indicates bound probe. The actual size of a nucleus is approximately 5 μm. The original magnification was ×630.

ic fluorochrome, was used as a counterstain.

To minimize the amount of probe DNA required, the procedure was scaled down to require only 1–2 × 10^4 nuclei and a hybridization volume of approximately 50 μl. To reduce loss of nuclei during the washing procedures, 10^7 mouse erythrocytes were added after hybridization. The erythrocytes were also cross-linked with DMS to prevent their disruption in the hypotonic buffers used in the immunological procedures. The addition of DMS-treated erythrocytes results in a large pellet, which contains the nuclei and can be handled conveniently.

Fluorescent micrographs of nuclei that had been hybridized with probe in suspension and then centrifuged onto microscope slides show that binding of the mouse satellite probe is specific and localized to the heterochromatic regions in the nuclei (Fig. 1). No binding of the human DNA probe was observed. Suspensions of nuclei hybridized in the absence of either probe showed no red fluorescence after immunofluorescent labeling procedures.

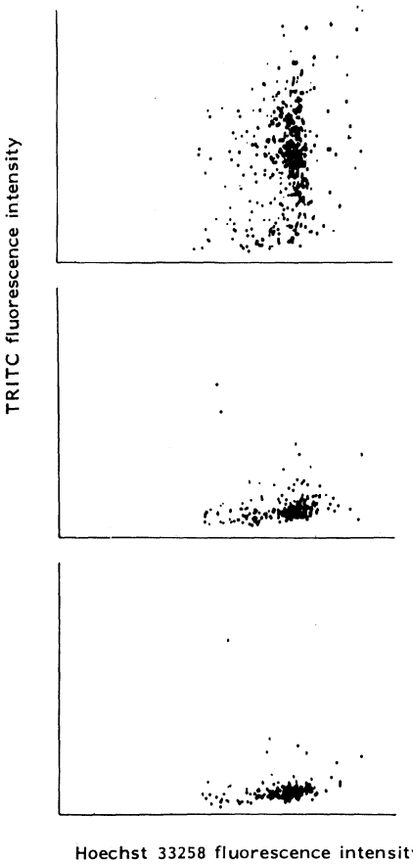

Fig. 2. Bivariate dot plots showing the TRITC fluorescence intensity and the Hoechst 33258 fluorescence intensity of nuclei hybridized in suspension and measured in a dual beam flow cytometer (*20*). Each dot represents the measurements of a single nucleus. Nuclei were hybridized with mouse satellite DNA probe (top); with a heterologous human DNA probe (middle); or in the absence of either probe (bottom). All three suspensions were subjected to immunofluorescent labeling procedures. Machine settings were identical during the measurements shown in all panels.

Bivariate dot plots of the TRITC versus Hoechst fluorescence intensity of nuclei hybridized in suspension and measured in a dual beam flow cytometer are shown in Fig. 2. In a dual beam flow cytometer, cells pass in single file through two lasers, which are tuned to emit light at different wavelengths (*22*). For each cell, the fluorescence signals emitted at each excitation beam can be quantified separately by means of a combination of spatial and chromatic filtering. Individual nuclei were identified at the first laser by the fluorescence of the DNA stain, Hoechst 33258. Added erythrocytes did not interfere with the flow cytometric measurement of hybridized probe; they contain no DNA and bound no Hoechst dye. Only single nuclei, selected on the basis of Hoechst fluorescence intensity, were analyzed for TRITC fluorescence at the second laser; erythrocytes and clumps of nuclei were disregarded.

The median TRITC fluorescence intensity of the nuclei was determined from the cumulative frequency curves (Fig. 3). The median fluorescence intensity of nuclei hybridized with mouse satellite DNA was approximately 20 times higher than that of nuclei hybridized with the human DNA probe (average of five separate experiments; range, 17 to 31 times). This value was corrected for the background signals measured in nuclei receiving antibody only. The fluorescence intensity of nuclei hybridized with the human DNA probe was only slightly higher than that of nuclei hybridized in the absence of probe, with or without antibody treatment.

The procedure for hybridization in suspension differs from the protocol for slides (*9*) in the reduced number of wash steps. This did not result in nonspecific antibody binding or nonspecific hybridization. As on slides, the specificity of the labeling depends on the stringency of the hybridization conditions. Other conditions tested gave less satisfactory results than the protocol presented here. For example, nuclei hybridized at 60°C in 2× standard saline citrate (SSC) without formamide showed high levels of nonspecific hybridization.

With flow cytometry, the fluorescence signal of the hybridized DNA probe can be more rapidly and accurately quantified than presently possible with microscope-based analysis of slides. The cell-to-cell variation in FITC fluorescence intensity is large; the coefficient of variation of the fluorescence distribution is approximately 25 to 30 percent. Variations of the same order of magnitude have been observed on slides by means of fluorescence hybridization (*14*) and with grain-counting or gamma-counting after autoradiographic hybridization (*23, 24*), although these techniques are capable of detecting smaller target sequences than the present method. We expect that attempts to reduce heterogeneity in hybridization efficiency and antibody labeling and to increase the accuracy of the flow cytometric measurement will lead to a reduction in this variability. However, since large numbers of nuclei can be analyzed individually and rapidly, the average hybridization signal of a cell population can be accurately determined, and the distribution and relative amount of a DNA sequence in a heterogeneous population can be studied. Since the median of the fluorescence distribution of nuclei hybridized with a probe complementary to 10 percent of the total genome was approximately 20 times higher than that of nuclei hybridized with a heterologous probe, DNA sequences occurring at frequencies as low as 1 percent should be readily detectable with the present techniques. By means of fluorescence hybridization on slides, it has recently been shown that the fluorescence intensity of hybridized human-specific probe is proportional to the amount of human DNA in interphase cell nuclei of human-hamster hybrids (*14*). The stoichiometry of the hybridiza-

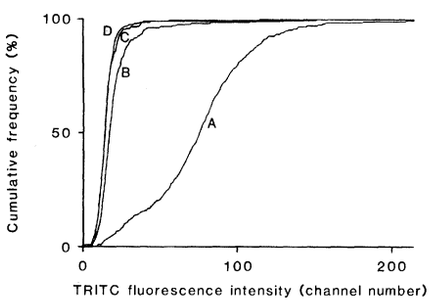

Fig. 3. Cumulative frequency curves of the TRITC fluorescence intensity of nuclei hybridized in suspension (*20*). The ordinate gives the sum of the number of nuclei with a TRITC fluorescence intensity less than or equal to the channel number indicated on the abscissa. The sum is expressed as a percentage of the total number of nuclei analyzed in each group. Mouse thymocyte nuclei hybridized with mouse satellite DNA probe (A), with human DNA probe (B), with no probe (C). The nuclei in (A), (B), and (C) were subjected to antibody labeling procedures. These groups are the same as those in Fig. 2. Mouse nuclei in (D) were not treated with antibodies.

tion reaction combined with further improvements in labeling and flow cytometric analysis may allow the rapid detection and quantification in interphase nuclei of individual chromosomes with chromosome-specific DNA sequence probes (*25–27*), amplified genes, or viral sequences.

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20. *Nuclei isolation and dimethylsuberimidate (DMS) treatment*: Nuclei were isolated from the thymocytes of 6- to 8-week-old BC3 female mice as described (*15–17*). For DMS treatment, K_2CO_3 and DMS were added (from a 5× concentrated stock solution, mixed immediately before use) to a suspension containing 5×10^6 nuclei/ml. Final K_2CO_3 and DMS concentrations were 20 m*M* and 3 m*M*, respectively. After 15 minutes at 25°C, the *p*H was adjusted from 10 to 8.0 by the addition of 50 μl of 100 m*M* citric acid per milliliter of suspension. Nuclei were washed once by centrifugation (300*g*, 10 minutes, 4°C) in IB (50 m*M* KCl and 5 m*M* Hepes buffer, at *p*H 8.0) containing 10 m*M* $MgSO_4$ (IB + M). *In situ hybridization in suspension*: To 2×10^4 DMS-treated nuclei [130 ng total DNA (*28*)] suspended in 20 μl IB in 1.5 ml Eppendorf tubes, the following solutions were added: 28 μl 100 percent formamide, 5.6 μl 20× SSC, 2 μl of a solution of salmon sperm DNA (10 mg/ml), and 1 μl AAF-modified DNA probe in 10 m*M* tris-HCl, 1 m*M* EDTA, at *p*H 7.6. The stock solutions of AAF-modified mouse satellite DNA isolated from liver nuclei (*9*) and of AAF-modified human DNA isolated from placenta contained 265 ng and 250 ng DNA/μl, respectively, resulting in a probe to complementary sequence ratio of 20:1. The mixtures of nuclei and probe were denatured by incubation at 70°C for 10 minutes. Hybridization was carried out at 37°C for 16 hours. Nuclei were washed by the addition of 1.0 ml hybridization buffer (50 percent formamide, 2× SSC, and 20 m*M* KCl, in 2 m*M* Hepes buffer) at 25°C and 100 μl DMS-treated mouse erythrocytes (RBC, see below). After 15 minutes agitation at 25°C, the nuclei were centrifuged for 2 minutes (8000*g*). The pellet was resuspended in 1.5 ml hybridization buffer by repeated syringing through a 23-gauge needle on a 1-ml syringe, centrifuged, and washed in 1.5 ml of IB + M. *Immunofluorescent labeling*: After hybridization, the nuclei were incubated for 60 minutes at 25°C in 200 μl of IB + M containing 2 percent normal goat serum (NGS; Nordic, Tilburg, The Netherlands) and a 1:500 dilution of a rabbit anti-AAF (*21*). The nuclei were washed once in IB + M and then incubated 60 minutes at 25°C in 200 μl of IB + M containing 2 percent NGS and a 1:50 dilution of a TRITC-conjugated goat anti-rabbit IgG (Nordic). The nuclei were washed once and then resuspended in 1.0 ml of IB + M by syringing five times. They were then filtered through 50-μm nylon mesh and stained with Hoechst 33258 (2 μg/ml). *DMS-treated erythrocytes*: Blood was collected into heparin by cardiac puncture from BC3 female mice. Washed erythrocytes (RBC), from which serum and white blood cells were removed by centrifugation, were suspended in physiological salt at a concentration of 10^8 per milliliter and treated three times with DMS. Final DMS concentrations during each treatment were 3 m*M*, 10 m*M*, and 10 m*M*. Additional adjustment of the *p*H to 9 to 10 with 100 m*M* K_2CO_3 was required during the last two treatments. The RBC were washed once in IB and resuspended in IB at a concentration of 10^8/ml. *Dual beam flow cytometry*: A FACS II flow cytometer modified for dual beam excitation, in which the fluorescent signals from the two beam spots are spatially and chromatically separated, was used. The nuclei and RBC were first illuminated by 300-mW UV light (multiline 351–364 from Spectra Physics laser model 171, Mountain View, Calif.) for Hoechst 33258 excitation. Hoechst fluorescence was measured through a KV418 (Schott) filter. Electronic gates were set to select single nuclei and exclude RBC, clumps, and debris from further analysis. The second laser (Coherent model CR 6) produced 100-mW light with a wavelength of 515 nm for TRITC excitation. The TRITC fluorescence of each particle identified as a nucleus by its Hoechst fluorescence was collected through a KV 580 (Schott) filter.
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A Human Y-Linked DNA Polymorphism and its Potential for Estimating Genetic and Evolutionary Distance

Abstract. *A human DNA sequence ($p12f_2$), derived from a partial Y-chromosome genomic library and showing homology with the X and Y chromosomes and with an undetermined number of autosomes, detected two Y-specific restriction fragment length variants on male DNA that had been digested with Taq I and Eco RI. These variants may have been generated through a deletion-insertion mechanism and their pattern of holoandric transmission indicates that they represent a two-allele Y-linked polymorphism (RFLP). By means of DNA from patients with inborn deletions in chromosome Y, this polymorphic DNA site was mapped to the interval Yq11.1–Yq11.22. The frequency of the rarest allele was about 35 percent in Algerian and Sardinian human males, whereas it was only 4 percent among Northern Europeans. The $p12f_2$ probe also detected Y-specific DNA fragments in the gorilla and chimpanzee. In view of the monosomy of the Y chromosome in mammalian species, Y-linked RFLP's may prove to be more useful than autosomal or X-linked markers in estimating genetic distances within and between species.*

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The application of restriction enzyme analysis and DNA recombinant technology to the study of human variation has uncovered a new class of genetic markers that are distributed among families and populations according to the rules of Mendelian inheritance [restriction fragment length polymorphisms (RFLP's)]. A total of 152 human RFLP's have been reported, 42 of which were detected with cloned genes and 110 with random nonrepeated genomic DNA sequences (*1*). This type of genetic variation has been found throughout the human genome with the exception of the Y chromosome (*2*). The recent construction of DNA libraries from the Y chromosome in mouse (*3*) and human (*4*) systems has facilitated the search for Y-linked RFLP's in these species. The detection of strain-specific restriction fragment variants by means of a Y-derived probe has been reported in the mouse (*5*). Our report describes two human Y-linked RFLP's and stresses their potential in studies of human population genetics and mammalian evolution.

The genomic human Y-DNA probe $p12f_2$ is a derivative of a p12f genomic probe that was previously isolated from a partial human Y-DNA library (*4*). Digestion of the p12f clone with Bgl II gave rise to a 2.3-kilobase (kb) fragment (12f2), which was isolated from 1 per-